Molecular Determinants of Modulation of \( \text{Ca}_v2.1 \) Channels by Visinin-like Protein 2*

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**Background:** Regulation of calcium channels by calcium sensor proteins mediates short-term synaptic plasticity.

**Results:** Visinin-like protein-2 (VILIP-2) increases facilitation of calcium channels through its N terminus, interlobe linker, and EF-hands 3 and 4.

**Conclusion:** Specific domains of VILIP-2 are responsible for regulation, including adjacent EF-hands that bind calcium.

**Significance:** These results reveal the molecular code used by calcium-sensor proteins to differentially regulate short-term synaptic plasticity.

\( \text{Ca}_v2.1 \) channels, which conduct P/Q-type \( \text{Ca}^{2+} \) currents, initiate synaptic transmission at most synapses in the central nervous system. \( \text{Ca}^{2+} \)-calmodulin-dependent facilitation and inactivation of these channels contributes to short-term facilitation and depression of synaptic transmission, respectively. Other calcium sensor proteins displace calmodulin (CaM) from its binding site, differentially regulate \( \text{Ca}_v2.1 \) channels, and contribute to the diversity of short-term synaptic plasticity. The neuronal calcium sensor protein visinin-like protein 2 (VILIP-2) inhibits inactivation and enhances facilitation of \( \text{Ca}_v2.1 \) channels. Here we examine the molecular determinants for differential regulation of \( \text{Ca}_v2.1 \) channels by VILIP-2 and CaM by construction and functional analysis of chimerae in which the functional domains of VILIP-2 are substituted in CaM. Our results show that the N-terminal domain, including its myristoylation site, the central \( \alpha \)-helix, and the C-terminal lobe containing EF-hands 3 and 4 of VILIP-2 are sufficient to transfer its regulatory properties to CaM. This regulation by VILIP-2 requires binding to the IQ-like domain of \( \text{Ca}_v2.1 \) channels. Our results identify the essential molecular determinants of differential regulation of \( \text{Ca}_v2.1 \) channels by VILIP-2 and define the molecular code that these proteins use to control short-term synaptic plasticity.

P/Q-type \( \text{Ca}^{2+} \) currents conducted by \( \text{Ca}_v2.1 \) channels initiate neurotransmitter release at most synapses in the central nervous system (1, 2). Short-term synaptic plasticity modulates neurotransmitter release in response to trains of repetitive action potentials, which often evoke facilitation followed by depression of the postsynaptic response (3–5). This regulatory mechanism encodes information in the frequency and pattern of action potentials and transmits it to the postsynaptic cell as a change in synaptic strength (3–5). \( \text{Ca}_v2.1 \) channels are required for synaptic facilitation at the Calyx of Held, a large, rapidly transmitting synapse in the auditory system (6). At that synapse, a component of short-term synaptic facilitation has been correlated with facilitation of P/Q-type calcium currents (6, 7), and the rapid phase of synaptic depression has been correlated with \( \text{Ca}^{2+} \)-dependent inactivation of P/Q-type calcium currents (8, 9).

In non-neuronal cells, \( \text{Ca}^{2+} \) influx through transfected \( \text{Ca}_v2.1 \) channels acts as a feedback regulator and causes facilitation followed by inactivation of the P/Q-type \( \text{Ca}^{2+} \) current in trains of repetitive depolarizations (10–14). \( \text{Ca}^{2+} \)-dependent facilitation and inactivation are mediated by binding of \( \text{Ca}^{2+} / \text{CaM} \) to a bipartite site in the C-terminal domain of \( \text{Ca}_v2.1 \) channels composed of an IQ-like motif (IM)² and a CaM-binding domain (CBD) (12, 14). Mutation of the IQ-like motif (IM-AA) abolishes \( \text{Ca}^{2+} \)-dependent facilitation, whereas deletion of the CBD (ΔCBD) reduces inactivation and has a lesser effect on facilitation (12, 14). Superior cervical ganglion neurons transfected with \( \text{Ca}_v2.1 \) channels show short-term synaptic facilitation and depression, and mutations of the IQ-like motif and CBD block facilitation and depression, respectively (15).

Short-term synaptic plasticity is diverse, with facilitation, depression, or facilitation followed by depression observed at different synapses in the central nervous system (3). As CaM is ubiquitously expressed, other proteins must determine the diversity of short-term synaptic plasticity. CaM is the founding member of a large family of CaS proteins, which are differentially expressed in neurons (16–18). Calcium-binding protein 1 (CaBP1) and visinin-like protein 2 (VILIP-2) differentially regulate \( \text{Ca}_v2.1 \) channels (19, 20). CaBP1 reduces facilitation and increases inactivation (19), whereas VILIP-2 slows the rate of inactivation and enhances facilitation (20). Mutation of the IQ-like motif (IM-AA) combined with the deletion of the CBD prevent VILIP-2 binding to \( \text{Ca}_v2.1 \) channels (20).

Both CaM and CaS proteins have four EF-hands connected by a central \( \alpha \)-helix (16–18). VILIP-2 and CaBP1 have large

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**Note:** This article contains supplementary Tables 1 and 2.

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The abbreviations used are: IM, IQ-like motif; CBD, CaM-binding domain; ΔCBD, deletion of the CBD; CaM, calmodulin; CaS, calcium sensor; CaBP1, calcium-binding protein 1; VILIP-2, visinin-like protein 2; MBP, maltose-binding protein; a.a., amino acids; PPR, paired pulse ratios.
N-terminal domains with a myristoyl lipid anchor (16–18). All four EF-hands of CaM bind Ca\(^{2+}\), whereas EF-hand 1 of VILIP-2 and EF-hand 2 of CaBP1 have changes in amino acid sequence that prevent high-affinity Ca\(^{2+}\) binding (16–18). In CaM regulation of Ca\(_{\text{v}}\),2.1 channels, local increases in Ca\(^{2+}\) lead to binding to the C-terminal lobe (EF-hands 3 and 4) and initiate facilitation by interaction with the IQ-like domain (11, 12, 14), whereas global increases in Ca\(^{2+}\) bind to the N-terminal lobe of CaM and mediate inactivation by interaction with the CBD (11, 12, 14). The domains of VILIP-2 responsible for differential regulation of Ca\(_{\text{v}}\),2.1 channels are unknown. In this study, we have constructed and analyzed chimeras of VILIP-2 and CaM to define the molecular basis for the differential regulation of Ca\(_{\text{v}}\),2.1 channels by VILIP-2. Our results show that the N-terminal domain up to the first EF-hand plus the C-terminal lobe of VILIP-2, together with its interlobe \(\alpha\)-helix, are both necessary and sufficient to confer VILIP-2-like regulatory properties on CaM. These results further define the molecular code that is used for CaS protein-dependent modulation of short-term synaptic plasticity.

**EXPERIMENTAL PROCEDURES**

**Construction of Chimeras**—We named the chimeras with a combination of two letters and four numbers. The letters are: N for the N-terminal domain including the myristoyl moiety and H for the interlobe helix. The four numbers correspond to the four EF-hands from N- to C-terminal. The VILIP-2 functional domains are highlighted in bold and underlined, whereas the CaM domains are in normal font. To exchange the functional domains of CaM and VILIP-2, overlapping PCR was used to swap the functional domains of VILIP-2 with those of CaM and inserted into the pcDNA3.1 vector at the 5’-Xhol and 3’-XbaI sites. The Pfam database (21) was used to determine the boundaries of CaM and VILIP-2 functional domains. The N12H34, N12H34, and N12H34 chimeras were generated by using overlapping PCR to fuse either the N terminus (a.a. 1–23), the N terminus to the end of EF-hand 1 (a.a. 1–55), EF-2 hand (a.a. 1–92), or helix (a.a. 1–96) of VILIP-2 and replaced the corresponding regions of CaM in pcDNA3.1. The N12H34 chimeras was used to generate N12H34 and N12H34. Using overlapping PCR, either the EF-hand 3 and EF-hand 4 (a.a. 97–end) or the helix, EF-hand 3, and EF-hand 4 (a.a. 93–end) of VILIP-2 replaced the corresponding segments of N12H34 and in pcDNA3.1.

**Cell Culture and Transfection**—Human embryonic kidney tsA-201 cells in DMEM/Ham’s F12 with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 100 units/ml penicillin and streptomycin (Invitrogen) were grown to 80% confluence at 37 °C and 10% CO\(_2\). Cells in 20-mm wells were transfected with cDNAs encoding Ca\(^{2+}\) channel subunits \(\alpha_2.1\) (2 \(\mu\)g), \(\beta_2\) (1.5 \(\mu\)g), \(\alpha\), \(\delta\) (1 \(\mu\)g), and VILIP-2 (1 \(\mu\)g) using TransIT-LT1 (Mirus Bio LLC, Madison, WI). cDNA encoding enhanced GFP was co-transfected to visualize the transfected cells (19). After 24 h, the cells were washed in serum-containing medium and subcultured on sterile 8-mm coverslips. Finally, 24–48 h after transfection, the cells were used for electrophysiological recordings.

**Electrophysiological Recordings and Data Analysis**—Whole-cell voltage clamp recordings were acquired from transfected tsA-201 cells at room temperature. The recordings were done in an extracellular solution containing (in mM) 10 CaCl\(_2\), or 10 BaCl\(_2\), 150 Tris, 1 MgCl\(_2\) (305 mosm) and with an intracellular solution consisting of (in mM) 120 N-methyl-D-glucamine, 60 Hepes, 1 MgCl\(_2\), 2 Mg-ATP, and 0.5 EGTA (295 mosm). The pH of both solutions was adjusted to pH 7.3 with methanesulfonic acid. Recordings were made with a HEKA EPC 10 patch clamp amplifier using PULSE software (HEKA Elektronik, Lambrecht, Germany) and filtered at 3 kHz. Leak and capacitive transients were subtracted using a P/4 protocol. Voltage protocols were adjusted to compensate for the more negative voltage dependence of activation in extracellular Ba\(^{2+}\) solution. Data analysis was performed using IGOR (WaveMetrics, Lake Oswego, OR). Activation curves were fit to determine values for the voltage of half-activation (\(V_{1/2}\)) and the slope (\(k\)) using the following Boltzmann equation: \(y = (y_{\text{max}} - y_{\text{min}})(1 + \exp(V_{1/2} - V)/k) + y_{\text{min}}.\) All average data represent the mean ± S.E. Statistical significance was determined using Student’s unpaired \(t\) test.

**Binding Assays**—MBP-VILIP-2 or MBP alone was immobilized on amylase beads (New England Biolabs, Beverly, MA) that were extensively washed with PBS buffer. The MBP proteins were incubated with 4 \(\mu\)g of CBD-GST, IM-GST, or GST alone proteins for 1 h at 4 °C. The binding buffer contained (in mM): 200 NaCl, 1 MgCl\(_2\), 20 Tris-HCl, and 1% Triton X-100. Ca\(^{2+}\) and EGTA were added as described under “Results.” After extensive washing, bound proteins were eluted at 97 °C with sample buffer (4×) and separated on a NuPAGE gel (Invitrogen). Immunoblotting was performed with monoclonal anti-GST (Sigma) or anti-MBP (New England Biolabs) antibodies. Blots were extensively washed with Tris-buffered saline with Tween-20. Analysis of the blots was done using the National Institutes of Health ImageJ program, and relative binding was normalized to control GST or MBP.

**RESULTS**

**Modulation of Ca\(_{\text{v}}\),2.1 Channels by CaM and VILIP-2**—CaM and CaS proteins are modular, composed of four EF-hand motifs separated by a central \(\alpha\)-helix (Fig. 1A). All four EF-hands of CaM are active in binding Ca\(^{2+}\), whereas EF-hand 1 of VILIP-2 is inactive (16–18). In addition, VILIP-2 has an extended N-terminal domain with a myristoyl lipid anchor (16–18). These molecular differences in VILIP-2 must be responsible for its unique regulatory properties, but the molecular determinants of differential regulation remain unknown. Chimeras of VILIP-2 and CaM were constructed in which the structural domains of VILIP-2 were substituted in CaM individually or in combinations (Fig. 1A). In this experimental strategy, the transferred domains from VILIP-2 confer VILIP-2-like regulatory properties on the chimeras, and overexpression of the chimeras displaces endogenous CaM and induces VILIP-2-like regulation of Ca\(_{\text{v}}\),2.1 channels. Overexpression of CaM itself does not have any functional effect, suggesting that it is already at a saturating concentration (19). Therefore, distinctive regulatory effects conferred by expression of the chimeras can be ascribed to molecular differences in the chimeras and
Modulation of CaV2.1 Channels by VILIP-2

**Figure 1. Modulation of CaV2.1 channels by CaM and VILIP-2.** A, schematic drawing of CaM/VILIP-2 chimeras. EF-hands are indicated by rectangles, and the central α-helix is indicated by a spiral. Filled rectangles, CaM EF-hands; striped rectangles, VILIP-2 EF-hands that bind Ca2+ with high affinity; open rectangles, VILIP-2 EF-hands that do not bind Ca2+ with high affinity. B, mean normalized Ica (n = 20) and Iba (n = 20) evoked by a 1-s depolarizing test pulse from −80 to +20 or +10 mV, respectively. C, mean ratio of residual current amplitude measured at the end of the 1-s pulse (IRes) over peak current amplitude (Ipeak) from CaV2.1-transfected cells in Ca2+ (black) and Ba2+ (white). D, mean normalized Ica (n = 10) and Iba (n = 9) from cells transfected with CaV2.1 and VILIP-2. E, mean ratio of Iba over Ica from CaV2.1 and VILIP-2-transfected cells in Ca2+ (black) and Ba2+ (white). F, mean normalized Ica (n = 19) and Iba (n = 12) during 100-Hz train of 5-ms pulses from −80 to +20 or +10 mV, respectively. G, paired pulse ratios (PPR) from tail currents evoked by test pulses from −80 to +30 mV for Ica (black; n = 25) or +20 mV for Iba (white; n = 13) without a prepulse (P1) or with a 50-ms prepulse to +10 mV (P2).

Ca2+/CaM-dependent inactivation of CaV2.1 channels was examined in transfected tsA-201 cells by application of a 1-s test pulse from −80 to 20 mV. Evoked Ca2+ currents inactivated rapidly when compared with currents recorded after replacing Ca2+ with Ba2+ as the charge carrier (Fig. 1, B and C). This Ca2+-dependent component of inactivation is caused by Ca2+/CaM interaction with the CBD in the C-terminal domain of CaV2.1 channels (10–12, 14). Co-expression of VILIP-2 with CaV2.1 prevented the Ca2+-dependent component of inactivation of CaV2.1 channels (Fig. 1, D and E).

To analyze Ca2+/CaM regulation in response to more physiological stimuli, Ca2+ and Ba2+ currents were recorded during a train of repetitive depolarizations to 20 mV for 5 ms at 100 Hz. Ca2+ currents were facilitated by the first depolarizations and then progressively inactivated (Fig. 1A). Both Ca2+-dependent facilitation and Ca2+-dependent inactivation were prevented when Ba2+ was the charge carrier (Fig. 1A). Ca2+-dependent facilitation was also observed when CaV2.1 tail currents were measured to assess conductance of CaV2.1 channels following paired depolarizations to 30 mV for 8 ms with or without a prepulse to 10 mV for 50 ms (Fig. 1G). In the presence of VILIP-2, repetitive depolarizations resulted in enhanced Ca2+-dependent facilitation and slowed Ca2+-dependent inactivation in cells expressing CaV2.1 channels (Fig. 1H) when compared with endogenous CaM (Fig. 1F). VILIP-2 induced Ca2+-dependent facilitation of the CaV2.1 tail current in a paired-pulse protocol (Fig. 1I), but the magnitude of Ca2+-dependent facilitation was not significantly different from CaM (Fig. 1, G and I). These effects of VILIP-2 on Ca2+-dependent facilitation and inactivation of CaV2.1 channels are consistent with a model in which VILIP-2 displaces CaM and regulates CaV2.1 channels by slowing Ca2+-dependent inactivation and enhancing Ca2+-dependent facilitation (20).

**Role of N-terminal Lobe of VILIP-2 in Ca2+-dependent Facilitation and Inactivation—CaM/VILIP-2 chimeras (Fig. 1A) were analyzed to examine which part of VILIP-2 mediates Ca2+-dependent facilitation. We included the N-terminal domain of VILIP-2 in all chimeras tested because it has been shown that deletion of the myristoylation site from the N terminus of VILIP-2 prevents its differential regulation of CaV2.1 channels (22). We tested three chimeras of the N-terminal lobe of VILIP-2. The first one included the N-terminal domain with its myristoyl moiety plus EF-hand 1 (N12H34), the second one added EF-hand 2 (N12H34), and the third one included the interlobe helix (N12H34) (Fig. 1A). Similar to endogenous CaM (Fig. 1B), co-expression of N12H34 with CaV2.1 channels sup-

not to additive effects of CaM plus the chimera. All of the chimeras we constructed were functionally expressed because each of them had a clearly detectable effect on regulation of CaV2.1 channels (supplemental Tables 1 and 2). However, only one chimera was able to fully confer the regulatory properties of VILIP-2 as described below.

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H, mean normalized Ica + 20 mV (n = 10) and Iba + 10 mV (n = 10) during 100-Hz trains of pulses from CaV2.1 and VILIP-2-transfected cells. I, PPR from tail currents of Ica (black; n = 21) or Iba (white; n = 19) without a prepulse (P1) or with a 50-ms prepulse to +10 mV (P2) in cells transfected with CaV2.1 and VILIP-2. The data shown are averages ± S.E. Asterisks indicate a significant difference (***, p < 0.001).
ported normal Ca\(^{2+}\)-dependent inactivation (Fig. 2, A and G). In contrast, co-expression of either \(N12H34\) or \(N12H34\) with Ca\(_{v}2.1\) channels caused significantly faster inactivation of both \(I_{Ca}\) and \(I_{Ba}\) (Fig. 2, C, E, and G). Moreover, substitution of Ca\(^{2+}\) with Ba\(^{2+}\) did not slow inactivation for \(N12H34\) (Fig. 2, C and G). Thus, the N-terminal lobe of VILIP-2 increases the rate of inactivation when substituted in CaM, opposite to the effect of intact VILIP-2.

A similar pattern of effects on inactivation was observed in trains of depolarizations (Figs. 2, B, D, and F). The \(N12H34\) chimera reduced facilitation when compared with CaM but induced similar Ca\(^{2+}\)-dependent inactivation of \(I_{Ca}\) during a train of depolarizations (Figs. 1F and 2B). In contrast, the \(N12H34\) and \(N12H34\) chimeras prevented facilitation in a paired-pulse protocol and during a train of depolarizations, as well as accelerated inactivation during trains of depolarizations, similar to their effects during single pulses (Fig. 2, D, F, and H). Taken together, these results suggest that the N-terminal lobe of VILIP-2 is not responsible for its increase in facilitation or its slowing of inactivation because transfer of its EF-hands to CaM has the opposite effect on channel regulation.

**Role of C-terminal Lobe of VILIP-2 in Ca\(^{2+}\)-dependent Facilitation and Inactivation**—To test whether facilitation is mediated by interaction of the C-terminal lobe of VILIP-2 with Ca\(_{v}2.1\) channels, we constructed a CaM/VILIP-2 C-terminal lobe chimera (\(N12H34\); Fig. 1A). Co-expression of \(N12H34\) with Ca\(_{v}2.1\) produced Ca\(^{2+}\)-dependent inactivation similar to endogenous CaM during single depolarizations (Figs. 1, B and C, and 3, A and B). However, in contrast to CaM (Fig. 1, F and G), the \(N12H34\) chimera blocked facilitation in a paired-pulse protocol and during trains of depolarizations, leaving only Ca\(^{2+}\)-dependent inactivation (Fig. 4, A and B). Thus, the VILIP-2 C-terminal lobe alone does not induce facilitation of Ca\(_{v}2.1\) channels when substituted in CaM chimeras.

To examine whether the addition of the interlobe helix of VILIP-2 is required for Ca\(^{2+}\)-dependent facilitation of Ca\(_{v}2.1\) channels, we tested a chimera in which the interlobe helix was added to the C-terminal lobe of VILIP-2 (\(N12H34\); Fig. 1A). \(N12H34\) prevented Ca\(^{2+}\)-dependent inactivation so that \(I_{Ca}\) and \(I_{Ba}\) had an identical, slow rate of inactivation (Fig. 3, C and D). The slow rate of inactivation in the presence of \(N12H34\) was identical to that induced by VILIP-2 (Fig. 3, E and F). In addition, \(N12H34\) caused Ca\(^{2+}\)-dependent facilitation in a paired-pulse protocol and during trains of depolarizations (Fig. 4, C and D, which was similar to VILIP-2 (Fig. 4E). Co-expression of VILIP-2 or the \(N12H34\) chimera with Ca\(_{v}2.1\) channels did not affect the voltage dependence of activation in response to different depolarizing membrane potentials (Fig. 4F). Taken together, the results from these chimeras indicate that the key elements required for the slowed Ca\(^{2+}\)-dependent inactivation and increased Ca\(^{2+}\)-dependent facilitation induced by VILIP-2 reside in the C-terminal lobe and the interlobe helix.

**Role of IQ-like Motif and CBD of Ca\(_{v}2.1\) Channels in Regulation by VILIP-2**—The IQ-like motif and the downstream CBD in the C-terminal domain of Ca\(_{v}2.1\) channels form the CaS protein regulatory site that is required for modulation by Ca\(^{2+}\) binding to endogenous CaM (14). Alanine substitution of the
These results indicate that the IM-AA mutation is not sufficient to induce facilitation of the CaV2.1/IM-AA channels. ICa currents evoked by single depolarizations or repetitive depolarizations at 100 Hz to assess the effects of VILIP-2 on CaV2.1/IM-AA channels. Ica conducted by CaV2.1/IM-AA channels had a rapid rate of inactivation, consistent with an intact Ca2+-dependent inactivation process as described previously. Co-expression of VILIP-2 or N12H34 blocked Ca2+-dependent inactivation and slowed the overall inactivation rate to the level observed with Ba2+ as current carrier. These results indicate that the IM-AA mutation is not sufficient to prevent the effect of VILIP-2 to inhibit Ca2+-dependent inactivation. In contrast, both VILIP-2 and N12H34 failed to induce facilitation of the CaV2.1/IM-AA channels. These results demonstrate that binding of VILIP-2 to the IQ-like domain of CaV2.1 channels is a primary requirement for Ca2+-dependent facilitation but not for Ca2+-dependent inactivation.

Deletion of the CBD has been shown to reduce Ca2+-dependent inactivation of CaV2.1 channels mediated by endogenous CaM (14). Unfortunately, this effect prevents testing the role of this site in VILIP-2 action because the effects of VILIP-2 would be occluded by the effects of the mutation. Thus, as expected, co-expression of VILIP-2 and N12H34 with CaV2.1 channels lacking the CBD domain (ΔCBD) had no effect on inactivation. To investigate the effects of VILIP-2 on facilitation of CaV2.1 channels lacking the CBD domain, we depolarized tsA-201 cells to +20 mV for 5 ms at a frequency of 100 Hz and recorded Ica. CaV2.1ΔCBD channels showed reduced facilitation and slower Ca2+-dependent inactivation than WT CaV2.1 channels (Figs. 1F and 6C). CaV2.1ΔCBD channels co-expressed with VILIP-2 showed similar facilitation and faster inactivation than CaV2.1 channels alone (Fig. 6F).
with VILIP-2 or N12H34 chimera showed increased facilitation and slowed inactivation of $I_{\text{Ca}}$ (Fig. 6, C and F). These results show that the CBD is not required for enhancement of facilitation of $Ca_v2.1$ channels by VILIP-2, consistent with previous results showing that it is not required for facilitation by endogenous CaM (14).
We further tested the site of VILIP-2 action in Ca\(^{2+}\)-dependent facilitation using the double mutant Ca\(_{\alpha}2.1\Delta CBD/IM-AA\). As expected, co-expression of VILIP-2 or the N12H34 chimera with Ca\(_{\alpha}2.1\Delta CBD/IM-AA\) had no effect on the slow rate of inactivation of \(I_{Ca}\) characteristic of mutants with the CBD deleted (Fig. 7, A, B, D, and E). However, this double mutation completely blocked facilitation induced by VILIP-2 during trains of depolarizations (Fig. 7, C and F), as expected from our results on Ca\(_{\alpha}2.1/IM-AA\) channels. These results confirm that the IM-AA mutation effectively blocks enhanced facilitation by VILIP-2. Taken together, the results from our studies of mutations of the IQ-like motif and CBD indicate that the IM residues in the IQ-like motif are required for Ca\(^{2+}\)-dependent facilitation but not for Ca\(^{2+}\)-dependent inactivation of Ca\(_{\alpha}2.1\) channels and that the CBD is not required for Ca\(^{2+}\)-dependent facilitation. These conclusions are consistent with previous findings on CaM regulation of Ca\(_{\alpha}2.1\) channels (14).

Binding of VILIP-2 to IQ-like Motif and CBD in Vitro—To test direct binding of VILIP-2 to the IQ-like motif, we used MBP-tagged VILIP-2 immobilized on amylose beads (Fig. 8A, Bait) and GST-tagged IM (Fig. 8A, Prey). We observed Ca\(^{2+}\)-independent binding of the GST-tagged IM domain (a.a. 1848–1964) to VILIP-2-MBP, suggesting stable interaction of these two proteins at basal Ca\(^{2+}\) levels (Fig. 8, A and B). These results indicate that the IQ-like motif should be dominant in VILIP-2 binding at low Ca\(^{2+}\) levels in resting cells. Surprisingly, the IM-AA mutation did not prevent VILIP-2 binding to the IQ-like motif in vitro (Fig. 8, C and D). These results explain how VILIP-2 can retain its inhibition of Ca\(^{2+}\)-dependent inactivation in the Ca\(_{\alpha}1.2/IM-AA\) mutant because it should displace CaM, whose binding is blocked by the IM-AA mutation (14), and thereby prevent Ca\(^{2+}\)-dependent inactivation as observed in Fig. 5, A and D. However, binding of VILIP-2 to Ca\(_{\alpha}2.1\) channels with the IM-AA mutation is not able to induce facilitation during trains of stimuli (Fig. 5, C and F).

Finally, we used MBP-tagged VILIP-2 immobilized on amylose beads (Fig. 9A, Bait) and GST-tagged CBD (Fig. 9A, Prey) to measure direct binding of VILIP-2 to the CBD. We observed Ca\(^{2+}\)-dependent binding of the GST-tagged CBD (a.a. 1959–2035) to VILIP-2-MBP, but only at 100 \(\mu M\) Ca\(^{2+}\) (Fig. 9, A and B). These results indicate that interaction of VILIP-2 with the CBD is likely to contribute to its overall binding affinity only during trains of stimuli that raise the concentration of free Ca\(^{2+}\) to this level near active zones where Ca\(_{\alpha}2.1\) channels are clustered (4, 5).

**DISCUSSION**

N-terminal Lobes of CaBP1 and VILIP-2 Enhance Ca\(^{2+}\)-dependent Inactivation—CaM and neuronal Ca\(_{\alpha}\) proteins contain four EF-hand motifs separated into two lobes by a central \(\alpha\)-helical domain. Ca\(_{\alpha}\) proteins differ from CaM in that they have an extended N-terminal domain, including an N-terminal myristoyl lipid anchor, and at least one EF-hand that cannot bind Ca\(^{2+}\). For example, EF-hand 1 of VILIP-2 and EF-hand 2 of CaBP1 do not bind Ca\(^{2+}\) (17, 23). The N-terminal lobe of CaM containing EF-hands 1 and 2 is primarily responsible for Ca\(^{2+}\)-dependent inactivation (12, 14). The N-terminal domain and EF-hand 2 of CaBP1 are sufficient to enhance inactivation in...
chimeras with CaM, suggesting that CaBP1 modulation of CaV2.1 channels is mediated by its unique EF-hand that is inactive in Ca2+/H11001 binding (24). Our results presented here show that transfer of the N-terminal lobe of VILIP-2 containing its EF-hands 1 and 2 increases inactivation of CaV2.1 channels in both Ca2+/H11001 and Ba2+/H11001 extracellular solutions (Figs. 2, C and G, and Fig. 10A). These results suggest that the N-terminal lobe containing the EF-hands that are inactive in Ca2+/H11001 binding (EF-hand 2 of CaBP1 and EF-hand 1 of VILIP-2) may actually be constitutively active in enhancing inactivation independent of Ca2+/H11001 in the context of the complete N-terminal lobes of these CaS proteins. The chimeric CaS proteins may be dominant over endogenous CaM in this respect. The ability of the N-terminal lobe to enhance inactivation is apparently suppressed in intact VILIP-2 by the presence of the C-terminal lobe of the protein plus the interlobe helix.

C-terminal Lobe of VILIP-2 Enhances Ca2+/H11001-dependent Facilitation—CaM induces Ca2+/H11001-dependent facilitation through interaction of its C-terminal lobe containing EF-hands 3 and 4 with the IQ-like motif of CaV2.1 channels (14). VILIP-2 enhances Ca2+/H11001-dependent facilitation of CaV2.1 channels (20). Our results show that the functional domain of VILIP-2 responsible for enhanced facilitation is the C-terminal lobe together with the interlobe helix (Fig. 10B). Thus, Ca2+/H11001-dependent facilitation is controlled by the C-terminal lobes of both CaM and VILIP-2. Chelation of Ca2+/H11001 by 10 mM EGTA blocks Ca2+/H11001-dependent inactivation but not Ca2+/H11001-dependent facilitation, consistent with activation of the facilitation process in response to brief, local increases in Ca2+/H11001 that cannot be intercepted by EGTA (11). These results suggest that Ca2+/H11001 influx through CaV2.1 channels generates a local increase in Ca2+/H11001, which promotes Ca2+/H11001 binding to the C-terminal lobe of preassociated CaM and initiates Ca2+/H11001-dependent facilitation by interaction with the IQ-like motif (Fig. 10C). In contrast, VILIP-2 binds to the IQ-like domain itself at low levels of Ca2+/H11001, prevents CaM binding, and enhances Ca2+/H11001-dependent facilitation when local Ca2+/H11001 levels rise (Fig. 10D). More global Ca2+/H11001 increases result in Ca2+/H11001 binding to the N-terminal lobe of CaM and initiation of Ca2+/H11001-dependent inactivation (Fig. 10C), which is prevented in the presence of VILIP-2 by its ability to bind at low levels of Ca2+/H11001 and displace CaM binding (Fig. 10D).

FIGURE 8. Binding of VILIP-2 to the IQ-like motif. A, Ca2+/H11001-independent binding of GST-tagged IM domain (Prey) (a.a. 1848–1964) with MBP-tagged VILIP-2 immobilized on amylose resin beads (Bait) in the presence of 2 μM Ca2+/H11001, 100 μM Ca2+/H11001, or 10 mM EGTA detected by immunoblot with anti-GST antibodies. B, normalized relative binding of the IM domain with VILIP-2. C, identical experiment to panel A but with the IM-AA mutant. D, normalized relative binding of the IM-AA domain with VILIP-2. The data shown are averages ± S.E. Asterisks indicate a significant difference (***, $p < 0.001$; **, $p < 0.01$).
Roles of CBD and IQ-like Motif in Regulation of CaV2.1 Channels by VILIP-2—Previous results showed that deletion of the CBD of CaV2.1 channels preferentially decreases Ca\(^{2+}\)/CaM-dependent inactivation and that mutation of the IQ-like motif preferentially reduces Ca\(^{2+}\)/CaM-dependent facilitation (14). As expected from these results, CaV2.1ΔCBD channels conduct Ca\(^{2+}\) currents that lack Ca\(^{2+}\)-dependent inactivation, and in this respect, they are functionally similar to WT CaV2.1 co-expressed with VILIP-2 (20). Co-expression of VILIP-2 with CaV2.1ΔCBD channels has no effect, but this is expected because these channels have no remaining Ca\(^{2+}\)-dependent inactivation for VILIP-2 to inhibit.

Ca\(^{2+}\)-dependent facilitation of CaV2.1ΔCBD channels was enhanced by VILIP-2 similarly to WT CaV2.1. These results are consistent with previous work showing that the Ca\(^{2+}\)/CaM-dependent facilitation is blocked by the IM-AA mutation and is less affected by deletion of the CBD (14). Taken together, these results suggest that enhanced facilitation of CaV2.1 channels by VILIP-2 is not mediated by its interaction with the CBD but do not allow a definite conclusion on the role of the CBD in the effects of VILIP-2 on Ca\(^{2+}\)-dependent inactivation.

It has previously been shown that binding of CaM to the IQ-like motif induces Ca\(^{2+}\)-dependent facilitation (12, 14). Our results indicate that VILIP-2 also binds to the IQ-like domain and that mutation of its first two residues (IM-AA) completely prevents enhancement of facilitation by VILIP-2. These results therefore suggest that the IQ-like domain is necessary for VILIP-2 modulation of Ca\(^{2+}\)-dependent facilitation. In contrast to these results, we did not observe any effect of the IM-AA mutation on reduction of Ca\(^{2+}\)-dependent inactivation in the presence of VILIP-2. These results match previous work in which the IM-AA mutation blocked Ca\(^{2+}\)/CaM-dependent facilitation but did not have a significant effect on Ca\(^{2+}\)-dependent inactivation (14). Overall, these results suggest that Ca\(^{2+}\)-dependent facilitation requires high-affinity binding of CaM or VILIP-2 to the IQ-like domain, which induces a conformational change that enhances facilitation in CaV2.1 channels but not in the CaV2.1/IM-AA mutant. In contrast, Ca\(^{2+}\)-dependent inactivation does not require binding to the IM domain and is retained in the IM-AA mutant, which therefore can still respond to VILIP-2 with slowed inactivation.

CaS Proteins and Short-term Synaptic Plasticity—Our previous results show that VILIP-2 and CaBP1 bind to the same regulatory site as CaM and differentially regulate CaV2.1 channels, favoring facilitation or inactivation, respectively (19, 20). Regulation of CaV2.1 channels expressed in synapses of sympathetic ganglion neurons by this mechanism causes synaptic facilitation and a rapid phase of synaptic depression (15). These results demonstrate that binding of Ca\(^{2+}\)/CaM to the IQ-like motif and CBD of CaV2.1 channels is sufficient to induce short-term synaptic plasticity and to determine its direction, i.e. facilitation versus depression. Neuronal calcium sensor-1 (NCS-1), which is closely related to VILIP-2, can enhance P/Q-type Ca\(^{2+}\)
currents in the Calyx of Held and can facilitate synaptic transmission in the Calyx of Held and in cultured hippocampal neurons (26, 27), but there is no evidence that it acts by directly binding to CaV2.1 channels. VILIP-2 and CaBP1 favor facilitation or depression, respectively, at sympathetic neuron synapses transfected with CaV2.1 channels and CaS proteins (28). These effects on short-term synaptic plasticity are blocked by mutation of the IQ-like motif and the CBD, demonstrating that direct regulation of CaV2.1 channels by binding of these neuronal CaS proteins can regulate short-term synaptic plasticity in a bidirectional manner. Because these CaS proteins can serve as a bidirectional switch and induce facilitation or depression of synaptic transmission, it is of great interest to understand the molecular basis for their differential regulation of CaV2.1 channels.

CaM is the most abundant CaS protein and is expressed at high levels in most eukaryotic cells (29, 30). VILIP-2 is mainly expressed in the caudate-putamen, neocortex, hippocampus, and cerebellum (17, 31). CaV2.1 is also present in those brain areas and is differentially localized in specific sets of synapses (32–35). Neuronal CaS proteins have higher affinities for Ca2+ than CaM (16, 25). Therefore, differential expression of these neospecific CaS proteins, together with their distinct affinities for binding to CaV2.1 channels, would fine-tune the neurotransmitter release and synaptic plasticity initiated by these channels (4). Our results presented here provide the initial insights into the molecular code for the differential regulation of CaV2.1 channels and synaptic plasticity by different neuronal CaS proteins. Evidently, domain-specific differences in amino acid sequence in these CaS proteins are both necessary and sufficient to define the regulatory mode of these crucial neuronal signaling proteins in short-term synaptic plasticity.

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