

Calretinin regulates Ca^{2+} -dependent inactivation and facilitation of $\text{Ca}_v2.1$ Ca^{2+} channels through a direct interaction with the $\alpha_12.1$ subunit*

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Running title: *Calretinin modulates $\text{Ca}_v2.1$ Ca^{2+} channels*

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Background: Ca^{2+} -dependent inactivation and facilitation of $\text{Ca}_v2.1$ Ca^{2+} channels are major determinants of neuronal excitability and synaptic plasticity.

Result: The Ca^{2+} -binding protein calretinin interacts with $\text{Ca}_v2.1$ and inhibits Ca^{2+} -dependent inactivation and enhances facilitation of $\text{Ca}_v2.1$.

Conclusion: In addition to its role as a diffusible Ca^{2+} buffer, calretinin can interact with targets such as $\text{Ca}_v2.1$ and modulate their function.

Significance: Calretinin- $\text{Ca}_v2.1$ interactions may shape Ca^{2+} signaling dynamics in neurons.

SUMMARY :

Voltage-gated $\text{Ca}_v2.1$ Ca^{2+} channels undergo dual modulation by Ca^{2+} , Ca^{2+} -dependent inactivation (CDI) and facilitation (CDF), which can influence synaptic plasticity in the nervous system. While the molecular determinants controlling CDI and CDF have been the focus of intense research, little is known

about the factors regulating these processes in neurons. Here, we show that calretinin (CR), a Ca^{2+} binding protein highly expressed in subpopulations of neurons in the brain, inhibits CDI and enhances CDF by binding directly to $\alpha_12.1$. Screening of a phage display library with CR as bait revealed a highly basic CR-binding domain (CRB) present in multiple copies in the cytoplasmic linker between domains II and III of $\alpha_12.1$. In pull-down assays, CR binding to fusion proteins containing these CRBs was largely Ca^{2+} -dependent. $\alpha_12.1$ coimmunoprecipitated with CR antibodies from transfected cells and mouse cerebellum, which confirmed the existence of CR- $\text{Ca}_v2.1$ complexes *in vitro* and *in vivo*. In HEK293T cells, CR significantly decreased $\text{Ca}_v2.1$ CDI and increased CDF. CR binding to $\alpha_12.1$ was required for these effects, since they were not observed upon substitution of the II-III linker of $\alpha_12.1$ with that from the $\text{Ca}_v1.2$ α_1 subunit ($\alpha_11.2$) which lacks the CRBs. In addition, coexpression of a protein containing the CRBs blocked the modulatory action of CR, most likely by competing with CR for interactions with $\alpha_12.1$. Our findings

highlight an unexpected role for CR in directly modulating effectors such as Ca_v2.1, which may have major consequences for Ca²⁺-signaling and neuronal excitability.

Voltage-gated Ca_v2.1 channels mediate Ca²⁺ influx that triggers neurotransmitter release from presynaptic nerve terminals at most synapses in the central nervous system (1). Because the amount of neurotransmitter released is proportional to the third or fourth power of the presynaptic Ca²⁺ concentration (2,3), modulation of Ca_v2.1 properties can greatly alter synaptic output and contribute to mechanisms of synaptic plasticity (4).

Like other Ca_v channels, Ca_v2.1 channels undergo a prominent feedback regulation by incoming Ca²⁺ ions, which depends on calmodulin (CaM) (5,6). However, unlike other Ca_v channels, CaM binding to the cytoplasmic C-terminal domain of α₁2.1 mediates both a negative and positive regulation by incoming Ca²⁺ ions, Ca²⁺-dependent inactivation (CDI) and facilitation (CDF) (7). Both CDI and CDF have been described for presynaptic Ca_v2.1 channels at central synapses (8-10) and can cause short-term depression and facilitation, respectively, of neurotransmitter release (11).

Ca²⁺-dependent modulation of native Ca_v2.1 channels is heterogeneous in neurons (12), which can be due to alternative splicing of sequences encoding the α₁2.1 C-terminal domain (13,14) or differences in auxiliary Ca_vβ subunit expression (15). In addition, neuron-specific Ca²⁺ binding proteins related to CaM can influence the extent of Ca_v2.1 CDF and CDI: CaBP1 inhibits CDF and enhances CDI (16), while VILIP-2 increases CDF and inhibits CDI (17). CaBP1 and VILIP-2 bind to the same sites as CaM in the C-terminal domain of α₁2.1, but structural differences from CaM underly their distinct modulation of Ca_v2.1 (17,18).

Within the EF-hand superfamily of Ca²⁺ binding proteins, CaM, CaBP1, and VILIP-2 are considered Ca²⁺ sensors, so defined by their abilities to undergo Ca²⁺-

dependent conformational change and interact with effectors. In contrast, Ca²⁺ binding proteins such as parvalbumin and calbindin D-28k generally act as diffusible Ca²⁺ buffers that modulate cytoplasmic Ca²⁺ signals (19). Like Ca²⁺ chelation by EGTA (7), parvalbumin and calbindin D-28k can significantly alter CDI of Ca_v2.1 channels in transfected HEK293T cells (20). However, as parvalbumin and calbindin D-28k were not reported to associate directly with α₁2.1, their actions on Ca_v2.1 CDI are likely to be indirect and due primarily to their actions as diffusible Ca²⁺ buffers (20).

Calretinin (CR) is a EF-hand Ca²⁺-binding protein that is highly expressed in cerebellar granule neurons where Ca_v2.1 channels are also expressed (19,21,22). Like parvalbumin and calbindin D-28k, CR is considered mainly as a Ca²⁺ signal modulator in neurons. Based on its Ca²⁺ chelating properties, CR can significantly shape the spatiotemporal properties of Ca²⁺ signals generated by plasma membrane and intracellular Ca²⁺ channels (23). Yet, CR undergoes large conformational changes upon binding to Ca²⁺ (24,25), which may mediate Ca²⁺-dependent interactions with integral membrane proteins (26). While investigating the potential targets of CR, we discovered a consensus CR-binding motif in α₁2.1. Ca²⁺-dependent binding of CR to this region nullifies CDI and enhances CDF in transfected HEK293T cells, which suggests that local tethering of CR to α₁2.1 is required for channel modulation. Our findings provide the first evidence that CR can directly alter Ca²⁺ signaling through interactions with effectors, thus raising new possibilities for how CR may modulate neuronal and network excitability (27,28).

EXPERIMENTAL PROCEDURES

cDNAs and molecular biology

The following Ca_v2.1 subunit cDNAs were used: α₁2.1 (rbA isoform (29)), β_{2A} (30), and α_{2δ} (31). GST-α₁2.1 fusion proteins containing one or more CR-binding (CRB)

motifs were generated by PCR and subcloning of the corresponding sequence in *EcoRI/XhoI* sites of pGEX-4T1 (GE Healthcare Biosciences, Piscataway, NJ). For the chimeric Ca²⁺ channel $\alpha_12.1-1.2$ subunit, amino acids 864 – 983 from rat brain $\alpha_12.1$ were replaced by amino acids 807 – 923 of rat brain $\alpha_11.2$ (rbCII, (32)). The corresponding DNA sequence of $\alpha_11.2$ was amplified by PCR and cloned into *AscI/SgrAI* sites of the $\alpha_12.1$ -containing expression plasmid. GFP-CR was generated by cloning a PCR fragment containing the cDNA coding for human CR (836 bp) in the plasmid pEGFP-C1 (Life Technologies, Grand Island, NY) using CR-specific primers comprising *EcoRI* and *BamHI* sites; the sequence of the insert was verified prior to use in experiments. For the expression construct containing the first 3 CRB motifs of $\alpha_12.1$ (mcherry-CRB1-3), the corresponding sequence (aa 899-953) and Kozak sequence was amplified from rat brain $\alpha_12.1$ by PCR and subcloned into *XhoI* and *HindIII* restriction sites of pEGFPN1-mcherry (a kind gift from S. England). All cDNA constructs were subject to sequencing prior to use in experiments.

Cell Culture and Transfection

HEK293 cells transformed with SV40 T-antigen (HEK293T) were maintained in DMEM (Life Technologies, Grand Island, NY) with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO₂. Cells were grown to 70- 80% confluence and transfected with Gene Porter reagent (Genlantis, San Diego, CA) or Fugene 6 (Promega, Fitchburg, WI) according to the manufacturer's protocols. For electrophysiology, cells were plated in 35 mm dishes and transfected with Ca_v2.1 subunit cDNAs: $\alpha_12.1$ (2 μ g), β_{2A} (1 μ g), and $\alpha_2\delta$ (1 μ g). pEGFPN1 or GFP-CR was cotransfected (1 μ g), which facilitated identification of transfected cells by fluorescence. For pull-down assays, cells were plated in 60 mm dishes and transfected with GFP-CR (6 μ g). For coimmunoprecipitation experiments, cells were plated in 60 mm dishes and transfected

with Ca_v2.1 subunits GFP- $\alpha_12.1$ (3.2 μ g), β_{2A} (1.6 μ g), and $\alpha_2\delta$ (1.6 μ g) and pEGFPN1 (1.6 μ g) or GFP-CR (1.6 μ g).

Phage display and binding assays

Purified human recombinant CR-coated 7.5 cm² plastic plates and the Ph.D.TM-7 Phage Display Peptide Library Kit (New England Biolabs Inc, Biocconcept, Allschwil, Switzerland) were used according to the manufacturer's protocol with 3 rounds of panning. The cDNA coding for the surface-exposed heptapeptides (part of the pIII coat protein) was extracted from *E.coli* strain ER2738, amplified by PCR, and subject to DNA sequencing (Microsynth, GmbH, Balgach, Switzerland).

GST- $\alpha_12.1$ fusion proteins containing one or more CR-binding (CRB) motifs were expressed in *E. coli* and purified on glutathione-Sepharose according to standard protocols. For experiments with fluorescently tagged purified CR, Alexa 488-labeled CR (CR*; 0-20 μ M) was incubated with GST - $\alpha_12.1$ fusion proteins or GST control (10 μ M) in binding buffer (in mM: 140 K-glutamate, 2 ATP, 1.25 MgCl₂, 20 PIPES, 0.4 EGTA; pH 7.2, adjusted with KOH). Ca²⁺-containing solutions included 1.6 mM CaCl₂ (Ca²⁺_{free}: ~300 μ M). Reactions were carried out at room temperature for 1 hour. Sample mixtures were applied to GST MultiTrap 4B (GE Healthcare) 96-well filter plates pre-packed with Glutathione-Sepharose. Bound CR*/ GST- $\alpha_12.1$ complexes were eluted with elution buffer (10 mM glutathione, 50 mM Tris-HCl, pH 8.0) and fluorescence was measured with a Victor X3 plate reader (Perkin Elmer, Waltham, MA).

For experiments with GFP-CR expressed in HEK293T cells, transfected cells were harvested and homogenized in 1 ml of ice-cold cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% TritonX-100, 0.25% w/v sodium deoxycholate, 1 mM EDTA, pH 7.4 and protease inhibitors) containing either 1.6 mM CaCl₂ or 1 mM EGTA. The homogenate was rotated at 4°C for 1 hour to solubilize

membrane proteins and the insoluble material was separated by centrifugation at 16,100 x g (30 min). The supernatant (300 µl) was incubated with 40 µl of a 50% slurry of immobilized GST-CRB1-3 brought to 1 ml with the lysis buffer at 4°C overnight. The beads were washed three times with 1 ml of ice cold lysis buffer and the bound proteins were eluted with SDS-containing sample buffer, subjected to SDS-PAGE, and transferred to nitrocellulose. Mouse monoclonal antibodies anti-GFP (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect bound GFP-CR by western blot.

Coimmunoprecipitation

For coimmunoprecipitation from transfected HEK293T cells, transfected cells were harvested 48 hours after transfection. Cell lysates were prepared as described in binding assays and incubated with 5 µg CR antibodies (Swant) and 40 µl protein A-Sepharose (50% slurry) overnight, rotating at 4°C. After three washes with 1 ml cell lysis buffer, proteins were eluted and analyzed by SDS-PAGE. Coimmunoprecipitated proteins were detected by Western blotting with anti- $\alpha_12.1$ antibodies (1:300, Alomone Labs, Jerusalem, Israel) and anti-GFP antibodies (1:3000, Santa Cruz Biotechnology).

For coimmunoprecipitation from mouse brain, the cerebellum was homogenized in 1 ml 250STMDPS buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM MgCl₂, 1 mM DTT, pH 7.4, and protease inhibitors). The nuclear fraction was removed by centrifugation at 800 x g for 15 min. The membrane fraction was separated from the cytosolic fraction by ultracentrifugation at 100,000 x g for 1 h. The membrane pellet was solubilized in 1 ml of solubilization buffer [Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5), 1% Triton X-100, and protease inhibitors] at 4°C for 30 min and insoluble material removed by ultracentrifugation at 100,000 x g for 1 h. Either 5 µg of rabbit IgG (Life Technologies) or anti-CR antibodies (Swant, Marly, Switzerland) were added to the solubilized membrane proteins along with 50

µl of Protein A-Sepharose (50% slurry, Sigma-Aldrich, St. Louis, MO). Reactions were continued overnight with end-over-end rotation at 4°C. The resin was collected by centrifugation and rinsed three times with 1 ml of solubilization buffer. Bound proteins were eluted, resolved by 4-12% SDS-polyacrylamide gel and western blotting with anti-CR antibodies (1:5000, Swant) and anti- $\alpha_12.1$ antibodies as described above.

Electrophysiology of transfected HEK293T cells

Whole-cell patch-clamp recordings were acquired 36-60 h post-transfection with a HEKA (Lambrecht/Pfalz, Germany) EPC-9 patch-clamp amplifier. External recording solution contained (in mM): 150 Tris, 1 MgCl₂, and 10 CaCl₂ or BaCl₂. Internal solution contained (in mM): 140 N-methyl-D-glucamine, 10 HEPES, 2 MgCl₂, 2 Mg-ATP, and 0.5 EGTA. The pH of both solutions was adjusted to 7.3 with methanesulfonic acid. Electrode resistances were 1-2 MΩ in the bath solution, and series resistance was ~2-4 MΩ, compensated 60- 80%. Membrane potential was held at -60 mV prior to action potential waveform protocols or to -80 mV prior to all other experiments. Acquired data was analyzed using Igor Pro software (Wavemetrics) and statistics were performed using SigmaPlot (Systat Software). All averaged data are presented as the mean ± SEM. In the figures, numbers of cells (n) are indicated in parentheses.

RESULTS

CR interacts with Ca_v2.1

While screening a heptapeptide phage display library for CR-interacting proteins, we identified a consensus CR-binding sequence, H(R/K)HRRR(E/D), consisting of 5-6 basic residues (His/Arg) flanked by an acidic (Glu/Asp) residue (Fig.1A). Bioinformatic analysis revealed that this CR-binding (CRB) sequence was repeated 5 times in the cytoplasmic loop between domains 2 and 3 of $\alpha_12.1$ (Fig.1B). The CRBs are C-terminal to the synaptic protein-interaction site

“synprint”; (33) and are highly conserved in $\alpha_{1.2.1}$ between species. The CRB-containing region is also present in the $\text{Ca}_v2.2$ α_1 subunit ($\alpha_{1.2.2}$, Supplementary Fig.1), but absent in Ca_v1 α_1 subunits. To test if the CRB(s) directly interact with CR, pull-down assays were performed with GST-fusion proteins containing the first 3 CRBs in $\alpha_{1.2.1}$ (GST-CRB1-3). CR bound to GST-CRB1-3, but not to control GST, in the presence of Ca^{2+} (1.6 mM). Binding of CR was Ca^{2+} -dependent since it was prevented when free Ca^{2+} was chelated with EGTA (1 mM, Fig.1C). To estimate the affinity of CR binding to $\alpha_{1.2.1}$, GST-CRB1-3 was incubated with varying amounts of fluorescently tagged CR. Binding was relatively low-affinity ($\text{EC}_{50} \sim 10 \mu\text{M}$) and was stronger with than without Ca^{2+} (Fig.1D). Compared to CR binding to a GST-fusion protein containing only the most N-terminal CRB (CRB1), CR binding in the linear range of the binding curve was increased by $19.6 \pm 7.9\%$ and $32.6 \pm 13.6\%$ for GST-proteins containing 2 (CRB 1,2) and 3 (CRB1-3) motifs, respectively (data not shown). These results confirm that CR binds in a Ca^{2+} -dependent manner to the CRBs in $\alpha_{1.2.1}$.

If CR is an interacting partner of $\text{Ca}_v2.1$, it should also associate with the intact channel. To test this prediction, we coexpressed CR and $\text{Ca}_v2.1$ in HEK293T cells and used CR antibodies in coimmunoprecipitation experiments. CR antibodies brought down $\alpha_{1.2.1}$ in cells cotransfected with $\text{Ca}_v2.1$ + CR. Some $\alpha_{1.2.1}$ was brought down non-specifically by CR antibodies since a small amount of $\alpha_{1.2.1}$ was detected in immunoprecipitations from cells transfected with $\text{Ca}_v2.1$ alone (Fig.1E). However, a larger signal corresponding to $\alpha_{1.2.1}$ was consistently detected when $\text{Ca}_v2.1$ was coexpressed with CR, indicating some specific association of CR with $\alpha_{1.2.1}$. To verify these results in the native context, we performed coimmunoprecipitation experiments with extracts from mouse cerebellum given that CR and $\text{Ca}_v2.1$ are both expressed highly in cerebellar granule neurons (19,21,22). In these experiments, CR

antibodies but not control IgG coimmunoprecipitated $\alpha_{1.2.1}$ (Fig.1F). Together, these results demonstrate that CR associates with recombinant and native $\text{Ca}_v2.1$ channels in the brain.

CR modulates CDI and CDF of $\text{Ca}_v2.1$

To investigate the functional consequences of CR interacting with $\alpha_{1.2.1}$, we compared the properties of $\text{Ca}_v2.1$ transfected alone or cotransfected with CR in HEK293T cells. Because $\text{Ca}_v2.1$ channels are strongly modulated by Ca^{2+} , we analyzed both Ca^{2+} and Ba^{2+} currents (I_{Ca} and I_{Ba} , respectively). In initial experiments, we found that CR had no significant impact on I_{Ca} or I_{Ba} current density or on voltage-dependent activation of I_{Ba} , although it caused a modest positive shift in the current-voltage (I-V) relation for I_{Ca} ($V_{1/2} = 2.7 \text{ mV} \pm 0.7 \text{ mV}$ for $\text{Ca}_v2.1$ alone vs. $7.3 \pm 0.4 \text{ mV}$ for $\text{Ca}_v2.1$ +CR, $p < 0.001$; $k = -5.4 \pm 0.2$ for $\text{Ca}_v2.1$ alone vs. -4.8 ± 0.1 for $\text{Ca}_v2.1$ +CR, $p = 0.03$, both by t-test; Fig.2A,B).

Ca^{2+} -dependent binding of CR to the channel could dynamically alter Ca^{2+} -dependent modulation of $\text{Ca}_v2.1$. Due to a reliance on global elevations in Ca^{2+} , CDI can be suppressed Ca^{2+} buffers such as EGTA (5-10 mM). In contrast, CDF, which depends on rapid, local Ca^{2+} elevations, is not blunted by high concentrations of EGTA (6,7). If CR acted as a Ca^{2+} buffer when bound to the channel, it could suppress CDI of $\text{Ca}_v2.1$. To test this, we performed experiments dissecting voltage- from Ca^{2+} -dependent inactivation (VDI and CDI, respectively). Voltage protocols consisted of 2 test pulses (P1, P2) separated by a conditioning prepulse (Pre) to varying voltages (Fig.3A). With both Ca^{2+} and Ba^{2+} as the charge carrier, the second test current is smaller than the first due to inactivation induced by the prepulse. The ratio of the P2:P1 test current amplitudes can therefore be used as a metric for inactivation (Fig.3B). For $\text{Ca}_v2.1$, I_{Ca} , inactivation is significantly stronger than for I_{Ba} (P2/P1 = 0.31 ± 0.07 for I_{Ca} vs. 0.68 ± 0.04 for I_{Ba} , $p < 0.001$). In addition, the P2:P1 ratio exhibits U-shaped dependence on prepulse voltage with a

minimum at the prepulse voltage evoking the maximal inward I_{Ca} (Fig.3B). In contrast, I_{Ba} shows a more modest and monotonic increase in inactivation with prepulse voltage due to VDI (Fig.3A,B). Since $Ca_v2.1$ current density positively affects CDI (14,20), we restricted analysis to cells exhibiting maximal current densities between 13 and 19 pA/pF.

Compared to the strong CDI in cells transfected with $Ca_v2.1$ alone, CDI was virtually undetectable across the full range of prepulse voltages in cells co-transfected with CR (Fig.3A-B). At prepulse voltages between -20 and +10 mV, I_{Ca} actually underwent facilitation ($P2/P1 > 1$). Facilitation was Ca^{2+} -dependent in that there was no effect of CR on inactivation of I_{Ba} (Fig.3C). Consistent with protocols that induce CDF (6), a prepulse to 0 mV induced a significant acceleration in activation kinetics of the P2 compared to the P1 current for both $Ca_v2.1$ alone ($\tau_{P1} = 6 \pm 1.3$ ms vs. $\tau_{P2} = 2 \pm 0.3$ ms, $n=6$, $p=0.02$) and $Ca_v2.1+CR$ ($\tau_{P1} = 12.4 \pm 1$ ms, vs. $\tau_{P2} = 1.9 \pm 0.2$ ms, $n=9$, $p < 0.001$). However, the prepulse-induced speeding of the P2 activation kinetics was significantly greater for cells cotransfected with CR compared to $Ca_v2.1$ alone (~125%, $p=0.001$). To quantitate the Ca^{2+} -dependent modulation seen with a 0-mV prepulse, the P2/P1 ratio of I_{Ba} was subtracted from that for I_{Ca} . This analysis clearly indicated that unlike $Ca_v2.1$ alone, which shows significant CDI, CR caused overt CDF (Fig.3C).

We further analyzed the effects of CR on $Ca_v2.1$ CDF using action potential (AP) waveforms. During a train of 200-Hz AP stimuli, CDF is evident as a progressive increase in the amplitude of I_{Ca} ; I_{Ba} undergoes less facilitation, which is voltage-dependent (Fig.4A,B). Consistent with our data obtained with conditioning prepulses (Fig.3), CR caused a significantly larger increase in I_{Ca} but not I_{Ba} by the end of the train (Fig.4C). We measured CDF as the difference in the amplitude of I_{Ca} and I_{Ba} at the end (0.5 s) of the train. By this metric, CDF for $Ca_v2.1$ was significantly greater with CR (0.22 ± 0.01 , $n=10$) than without (0.13 ± 0.03 , $n=8$; $p < 0.01$,

t-test). These results confirm that CR enhances CDF of $Ca_v2.1$.

These effects of CR on $Ca_v2.1$ CDI and CDF could be due to its actions as a freely diffusible Ca^{2+} buffer. Alternatively, CR binding to the $\alpha_12.1$ II-III linker may directly suppress CDI and enhances CDF. To distinguish between these possibilities, we took advantage of the fact that the CRBs present in $\alpha_12.1$ are not conserved in the $Ca_v1.2$ α_1 subunit ($\alpha_11.2$). If CR binding to $\alpha_12.1$ is necessary for $Ca_v2.1$ modulation, then chimeric channels in which the 2-3 linker of $\alpha_11.2$ is substituted for that in $\alpha_12.1$ ($Ca_v2.1-1.2$), should prevent effects of CR on CDI and CDF. Consistent with this prediction, $Ca_v2.1-1.2$ channels underwent CDI that was not affected by CR (Fig.5A-C). In addition, there was no significant difference in CDF during AP trains in cells transfected with $Ca_v2.1-1.2$ alone and cells cotransfected with CR ($p=0.57$; Fig.6). These results show that the CRBs are required for CR modulation of $Ca_v2.1$ CDI and CDF.

To verify the importance of CR binding to the CRBs for $Ca_v2.1$ modulation, we tested the impact of a peptide which should competitively displace CR from binding sites in the $\alpha_12.1$ II-III linker. Since our biochemical experiments indicated that CR binds to a peptide sequence including the first 3 CRBs (Fig.1), we generated a mcherry-tagged peptide containing this region (CRB1-3). Because CR had no effect on I_{Ba} either in inactivation or facilitation protocols (Fig.3,4), we restricted analysis to I_{Ca} and assumed effects on I_{Ca} inactivation and facilitation reflected effects on CDI and CDF, respectively. When cotransfected with $Ca_v2.1+CR$, CRB1-3 significantly opposed the decrease in CDI caused by CR (for a 0-mV prepulse, $P2/P1 = 1.0 \pm 0.05$ for $Ca_v2.1+CR$ vs. 0.58 ± 0.11 for $+CR+CRB1-3$; $p < 0.01$; Fig.7A). Coexpression of CRB1-3 also significantly inhibited the effect of CR on CDF by the end of a 1-s AP train ($p < 0.05$; Fig.7B). In contrast, there was no difference in CDI ($p=0.43$ for a 0-mV prepulse) or CDF ($p=0.93$ at the end of the 1-s train) in cells transfected with $Ca_v2.1$

alone and those cotransfected with Ca_v2.1+CRB1-3 (Fig.7C,D), which argued against non-specific inhibitory effects of CRB1-3 on Ca_v2.1 that were independent of CR modulation. Taken together, our results support a mechanism in which CR binding to the α₁2.1 II-III linker directly inhibits Ca_v2.1 CDI and enables enhanced CDF of Ca_v2.1 during repetitive stimuli.

DISCUSSION

Our study provides key evidence for a new role for CR as an integral component of Ca_v2.1 complexes that modulates Ca_v2.1 function. First, CR binds in a Ca²⁺-dependent manner to basic motifs in the α₁2.1 II-III linker. Second, CR forms a complex with Ca_v2.1 channels in the brain. Third, the interaction of CR with the α₁2.1 II-III linker inhibits CDI and enhances CDF of Ca_v2.1 channels and enhances CDF in transfected HEK293T cells. Because of the cellular overlap in CR and Ca_v2.1 expression in the brain (22,34), CR interactions with Ca_v2.1 should be considered in models of how this protein controls Ca²⁺ signaling and excitability in neurons.

Ca²⁺ dependent binding of CR to effectors

CR has long been considered a diffusible Ca²⁺ buffer which can alter spatiotemporal aspects of Ca²⁺ signaling (19,23). However, multiple lines of evidence suggest that CR may not be freely diffusible and may in fact, interact with proteins in a manner analogous to “Ca²⁺ sensors” such as CaM. First, CR undergoes large conformational changes upon binding to Ca²⁺ (24,25). Similar Ca²⁺-dependent structural changes have been reported for the related EF-hand proteins, calbindin D28k and secretagoin (35,36). As in CaM, Ca²⁺ binding to CR may expose hydrophobic regions of the protein, which allow Ca²⁺-dependent interactions with other proteins. Second, in addition to its cytosolic localization, CR is also abundant in the membrane fraction of cerebellar extracts and less so under conditions of low Ca²⁺ (26). These latter findings could be explained by Ca²⁺-dependent interactions of CR with integral

membrane or membrane-associated proteins, much like the well-established role of CaM in regulating pre- and post-synaptic effectors (37).

Although our *in vitro* experiments indicate a relatively low binding affinity (~10 μM) of CR for the α₁2.1 CRB sequences in GST-CRB1-3 (Fig.1D), we believe this may underestimate the ability of CR to interact with α₁2.1 *in vivo*. Because we found greater CR binding to GST proteins containing 3 compared to 2 or 1 CRB sequences, it is likely that the 5 CRBs in the intact channel may increase the avidity of CR binding. In addition, CR is thought to be expressed at rather high concentrations in some neurons, ranging from approximately 40-80 μM (27,38). These concentrations of CR would be sufficient to saturate the CRB sequence(s) in α₁2.1 to a large extent, particularly if the affinity between CR and CRBs is even higher within the intact channel complex than *in vitro*. While our biochemical analyses cannot allow conclusions regarding the stoichiometry of CR binding to the α₁2.1 II-III linker, binding of CR to the GST-CRB1-3 protein was cooperative (Fig.1D). Since GST-CRB1-3 contains 3 of the 5 CRB sequences in the α₁2.1 II-III linker, it is possible that cooperativity in the binding assay resulted from multiple CR molecules binding to GST-CRB1-3. Alternatively, many EF-hand proteins including CR isoforms show reversible dimerization *in vitro* (39). The binding of CR dimers to the CRBs could therefore also lead to the apparent cooperativity in binding to GST-CRB1-3. Additional studies will be required to fully resolve the molecular thermodynamic properties of CR interactions with α₁2.1.

While our findings that CR coimmunoprecipitates with Ca_v2.1 channels in the brain (Fig.1F) clearly implicate Ca_v2.1 as a CR target, it is important to note that basic amino acid sequences similar to the α₁2.1 CRBs are likely present in other effectors. In particular, the CRBs are conserved in the “synprint” region of the Ca_v2.2 α₁ subunit (α₁2.2, Supp.Fig.1). While Ca_v2.2 channels do

not undergo CDF (40), synaptic protein interactions with the II-III linker of $\alpha_12.2$ regulate voltage-dependent inactivation of $\text{Ca}_v2.2$ and inhibition of these channels by heterotrimeric G-proteins (41). Thus, it is possible that CR binding to the CRBs in $\alpha_12.2$ could have important consequences for the modulation of $\text{Ca}_v2.2$ channels in neurons. The characterization of other CR-interacting proteins other than $\text{Ca}_v2.1$, and how they may be modulated by CR, is crucial for fully understanding Ca^{2+} signaling dynamics in the neuronal and non-neuronal cell-types in which CR is expressed (42,43).

Ca_v2.1 CDI/CDF modulation by CR

Significant progress has been made in elucidating the mechanisms underlying $\text{Ca}_v2.1$ CDI and CDF (see (44) for review). CDI depends on the N-terminal lobe of CaM, which responds to global rather than local Ca^{2+} signals. CDF depends on the C-lobe of CaM, which likely binds local Ca^{2+} ions as they emerge from the channel pore (6,45). In support of this model, intracellular dialysis with EGTA (10 mM) prevents CDI but not CDF (7). Moreover, CDF but not CDI is observed at the single channel level (46). This latter result and the findings that CDI increases with $\text{Ca}_v2.1$ current density (14,20) illustrate that CDI depends on Ca^{2+} influx through multiple open channels and so should be sensitive to Ca^{2+} buffering. Based on the ability of CR to rapidly depress presynaptic Ca^{2+} signals (47), it is assumed that CR will act as a Ca^{2+} buffer when faced with a rise in intracellular Ca^{2+} . Therefore, it is perhaps not surprising that coexpression of CR with $\text{Ca}_v2.1$ inhibited CDI. The remarkable result is that CR accomplishes this through its association with the $\alpha_12.1$ II-III linker (Figs. 6,7). Our biochemical and electrophysiological experiments show that disabling CR interactions with the $\alpha_12.1$ II-III linker prevents the channel modulation. The tethered CR may rapidly suppress global Ca^{2+} elevations that support CDI, which subsequently enhances CDF during trains of AP waveforms (Figs.3,4). Alternatively, CR binding to the II-III linker could allosterically

modulate CDI in the manner of a Ca^{2+} sensor. Despite the presence of molecular determinants for CDI and CDF in the C-terminal domain of $\text{Ca}_v \alpha_1$ subunits, auxiliary $\text{Ca}_v\beta$ subunit interactions with the I-II linker have been shown to modulate CDI and CDF of $\text{Ca}_v1.2$ channels (48). Experimental dissection of a Ca^{2+} buffering vs. Ca^{2+} sensor mechanism is complicated by the possibility that disrupting the Ca^{2+} buffering activity of CR might also negate its ability to interact with the channel, but nevertheless is an important goal for future studies.

It is noteworthy that the CRBs in the $\alpha_12.1$ II-III linker overlap with the bipartite synprint site in $\alpha_12.1$. Interactions between SNAREs and the synprint are thought to promote efficient coupling of Ca_v2 channels and exocytosis in presynaptic nerve terminals based on evidence that peptides containing the synprint site impair neurotransmission (49,50). Our findings suggest that such peptides may also influence CR regulation of $\text{Ca}_v2.1$ CDI and CDF and so may affect synaptic transmission via multiple mechanisms. In addition, splice variants lacking portions of the CRB/synprint region have been identified in neuroendocrine cells and various brain regions (51). The inability of CR to modulate CDI/CDF of such variants may further diversify $\text{Ca}_v2.1$ Ca^{2+} signaling between neuronal sub-types.

Neurophysiological significance of CR/Ca_v2.1

Immunohistochemical analyses indicate a number of neuronal cell groups in which CR and $\text{Ca}_v2.1$ colocalize. $\text{Ca}_v2.1$ channels are the major presynaptic Ca^{2+} channels in the nerve terminals forming the Calyx of Held synapse in the auditory brainstem (9,52). CR is detected presynaptically at these synapses but only at significant levels (>18% in rats) after postnatal day 14 (53). Electrophysiological recordings at the Calyx of Held synapse, usually done in brainstem slices from juvenile rats (postnatal day 8-10), indicate that the presynaptic $\text{Ca}_v2.1$ channels undergo CDI and CDF (8-10,54). Given our findings that CR inhibits CDI and enhances CDF, the developmental increase in CR would be

expected to promote activity-dependent Ca^{2+} influx that may limit synaptic depression and/or increase reliability in the mature Calyx of Held synapse(55).

As our coimmunoprecipitation of $\text{Ca}_v2.1$ with CR from mouse cerebellum would indicate, CR/ $\text{Ca}_v2.1$ complexes may play a role in cerebellar granule cells, the predominant cell-types expressing CR in the cerebellum (56,57). Granule cells provide the major excitatory drive to Purkinje neurons in the form of parallel fibers. Genetic inactivation of CR in mice increases the intrinsic excitability of granule cells and Purkinje cell firing rate *in vivo* (27,28,58). With respect to $\text{Ca}_v2.1$ in granule cells, loss of CR should inhibit $\text{Ca}_v2.1$ Ca^{2+} influx by enhancing CDI (Fig.3). Decreased I_{Ca} may seem at odds with the hyperexcitable phenotype of $\text{CR}^{-/-}$ granule cells, since it would be expected to limit activation of Ca^{2+} -

activated BK channels and subsequently, oppose repolarization following an action potential. However, we have observed compensatory changes in $\text{Ca}_v2.1$ subunit expression in cerebellar Purkinje neurons from mice lacking parvalbumin and calbindin (15), which could also explain the lack of correlation between our findings and that expected in $\text{CR}^{-/-}$ granule cells. In addition, $\text{Ca}_v2.1/\text{CR}$ interactions may be more relevant presynaptically, where enhanced CDF may support residual Ca^{2+} in parallel fiber terminals that causes short-term synaptic plasticity at the parallel fiber-Purkinje cell synapse (59).

In summary, our results implicate CR as a novel modulator of $\text{Ca}_v2.1$ channels, which may foreshadow yet additional roles for CR in actively regulating neuronal excitability and synaptic transmission through direct Ca^{2+} -dependent interactions with other effectors.

REFERENCES

1. Catterall, W. A. (2000) Structure and regulation of voltage-gated Ca^{2+} channels. *Annu Rev Cell Dev Biol* **16**, 521-555
2. Dodge, J., F.A., and Rahamimoff, R. (1967) Co-operative action of calcium ions in transmitter release at the neuromuscular junction. *J.Physiol.(Lond.)* **193**, 419-432
3. Augustine, G. J., and Charlton, M. P. (1986) Calcium dependence of presynaptic calcium current and post-synaptic response at the squid giant synapse. *J.Physiol.* **381**, 619-640
4. Catterall, W. A., and Few, A. P. (2008) Calcium channel regulation and presynaptic plasticity. *Neuron* **59**, 882-901
5. Lee, A., Wong, S. T., Gallagher, D., Li, B., Storm, D. R., Scheuer, T., and Catterall, W. A. (1999) Ca^{2+} /calmodulin binds to and modulates P/Q-type calcium channels. *Nature* **399**, 155-159
6. DeMaria, C. D., Soong, T., Alseikhan, B. A., Alvania, R. S., and Yue, D. T. (2001) Calmodulin bifurcates the local Ca^{2+} signal that modulates P/Q-type Ca^{2+} channels. *Nature* **411**, 484-489
7. Lee, A., Scheuer, T., and Catterall, W. A. (2000) Ca^{2+} /calmodulin-dependent facilitation and inactivation of P/Q-type Ca^{2+} channels. *J Neurosci* **20**, 6830-6838
8. Cuttle, M. F., Tsujimoto, T., Forsythe, I. D., and Takahashi, T. (1998) Facilitation of the presynaptic calcium current at an auditory synapse in rat brainstem. *J Physiol* **512**, 723-729
9. Forsythe, I. D., Tsujimoto, T., Barnes-Davies, M., Cuttle, M. F., and Takahashi, T. (1998) Inactivation of presynaptic calcium current contributes to synaptic depression at a fast central synapse. *Neuron* **20**, 797-807
10. Borst, J. G., and Sakmann, B. (1998) Facilitation of presynaptic calcium currents in the rat brainstem. *J Physiol* **513**, 149-155
11. Mochida, S., Few, A. P., Scheuer, T., and Catterall, W. A. (2008) Regulation of presynaptic $\text{Ca}_v2.1$ channels by Ca^{2+} sensor proteins mediates short-term synaptic plasticity. *Neuron* **57**, 210-216

12. Chaudhuri, D., Alseikhan, B. A., Chang, S. Y., Soong, T. W., and Yue, D. T. (2005) Developmental activation of calmodulin-dependent facilitation of cerebellar P-type Ca^{2+} current. *J Neurosci* **25**, 8282-8294
13. Chaudhuri, D., Chang, S. Y., DeMaria, C. D., Alvania, R. S., Soong, T. W., and Yue, D. T. (2004) Alternative splicing as a molecular switch for Ca^{2+} /calmodulin-dependent facilitation of P/Q-type Ca^{2+} channels. *J Neurosci* **24**, 6334-6342
14. Soong, T. W., DeMaria, C. D., Alvania, R. S., Zweifel, L. S., Liang, M. C., Mittman, S., Agnew, W. S., and Yue, D. T. (2002) Systematic identification of splice variants in human P/Q-type channel $\alpha_{12.1}$ subunits: implications for current density and Ca^{2+} -dependent inactivation. *J Neurosci* **22**, 10142-10152
15. Kreiner, L., Christel, C. J., Benveniste, M., Schwaller, B., and Lee, A. (2010) Compensatory regulation of $\text{Cav}2.1$ Ca^{2+} channels in cerebellar Purkinje neurons lacking parvalbumin and calbindin D-28k. *J Neurophysiol* **103**, 371-381
16. Lee, A., Westenbroek, R. E., Haeseleer, F., Palczewski, K., Scheuer, T., and Catterall, W. A. (2002) Differential modulation of $\text{Ca}_v2.1$ channels by calmodulin and Ca^{2+} -binding protein 1. *Nat. Neurosci.* **5**, 210-217
17. Lautermilch, N. J., Few, A. P., Scheuer, T., and Catterall, W. A. (2005) Modulation of $\text{Ca}_v2.1$ channels by the neuronal calcium-binding protein visinin-like protein-2. *J. Neurosci.* **25**, 7062-7070
18. Few, A. P., Lautermilch, N. J., Westenbroek, R. E., Scheuer, T., and Catterall, W. A. (2005) Differential regulation of $\text{Ca}_v2.1$ channels by calcium-binding protein 1 and visinin-like protein-2 requires N-terminal myristoylation. *J. Neurosci.* **25**, 7071-7080
19. Schwaller, B. (2009) The continuing disappearance of "pure" Ca^{2+} buffers. *Cell Mol Life Sci* **66**, 275-300
20. Kreiner, L., and Lee, A. (2006) Endogenous and exogenous Ca^{2+} buffers differentially modulate Ca^{2+} -dependent inactivation of $\text{Ca}_v2.1$ Ca^{2+} channels. *J Biol Chem* **281**, 4691-4698
21. Randall, A., and Tsien, R. W. (1995) Pharmacological dissection of multiple types of Ca^{2+} channel currents in rat cerebellar granule neurons. *J Neurosci* **15**, 2995-3012
22. Westenbroek, R. E., Sakurai, T., Elliott, E. M., Hell, J. W., Starr, T. V., Snutch, T. P., and Catterall, W. A. (1995) Immunohistochemical identification and subcellular distribution of the α_{1A} subunits of brain calcium channels. *J Neurosci* **15**, 6403-6418
23. Dargan, S. L., Schwaller, B., and Parker, I. (2004) Spatiotemporal patterning of IP_3 -mediated Ca^{2+} signals in *Xenopus* oocytes by Ca^{2+} -binding proteins. *J Physiol* **556**, 447-461
24. Schwaller, B., Durussel, I., Jermann, D., Herrmann, B., and Cox, J. A. (1997) Comparison of the Ca^{2+} -binding properties of human recombinant calretinin-22k and calretinin. *J Biol Chem* **272**, 29663-29671
25. Kuznicki, J., Wang, T. L., Martin, B. M., Winsky, L., and Jacobowitz, D. M. (1995) Localization of Ca^{2+} -dependent conformational changes of calretinin by limited tryptic proteolysis. *Biochem J* **308** (Pt 2), 607-612
26. Winsky, L., and Kuznicki, J. (1995) Distribution of calretinin, calbindin D28k, and parvalbumin in subcellular fractions of rat cerebellum: effects of calcium. *J Neurochem* **65**, 381-388
27. Gall, D., Roussel, C., Susa, I., D'Angelo, E., Rossi, P., Bearzatto, B., Galas, M. C., Blum, D., Schurmans, S., and Schiffmann, S. N. (2003) Altered neuronal excitability in cerebellar granule cells of mice lacking calretinin. *J Neurosci* **23**, 9320-9327
28. Schiffmann, S. N., Cheron, G., Lohof, A., d'Alcantara, P., Meyer, M., Parmentier, M., and Schurmans, S. (1999) Impaired motor coordination and Purkinje cell excitability in mice lacking calretinin. *Proc Natl Acad Sci U S A* **96**, 5257-5262
29. Stea, A., Tomlinson, W. J., Soong, T. W., Bourinet, E., Dubel, S. J., Vincent, S. R., and Snutch, T. P. (1994) The localization and functional properties of a rat brain α_{1A} calcium

- channel reflect similarities to neuronal Q- and P-type channels. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 10576-10580
30. Castellano, A., Wei, X., Birnbaumer, L., and Perez-Reyes, E. (1993) Cloning and expression of a neuronal calcium channel β subunit. *J Biol Chem* **268**, 12359-12366
 31. Starr, T. V. B., Prystay, W., and Snutch, T. P. (1991) Primary structure of a calcium channel that is highly expressed in the rat cerebellum. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5621-5625
 32. Snutch, T. P., Tomlinson, W. J., Leonard, J. P., and Gilbert, M. M. (1991) Distinct calcium channels are generated by alternative splicing and are differentially expressed in the mammalian CNS. *Neuron* **7**, 45-57
 33. Sheng, Z. H., Rettig, J., Takahashi, M., and Catterall, W. A. (1994) Identification of a syntaxin-binding site on N-type calcium channels. *Neuron* **13**, 1303-1313
 34. Schwaller, B., Meyer, M., and Schiffmann, S. (2002) 'New' functions for 'old' proteins: the role of the calcium-binding proteins calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies with knockout mice. *Cerebellum* **1**, 241-258
 35. Rogstam, A., Linse, S., Lindqvist, A., James, P., Wagner, L., and Berggard, T. (2007) Binding of calcium ions and SNAP-25 to the hexa EF-hand protein secretagogin. *Biochem J* **401**, 353-363
 36. Berggard, T., Miron, S., Onnerfjord, P., Thulin, E., Akerfeldt, K. S., Enghild, J. J., Akke, M., and Linse, S. (2002) Calbindin D28k exhibits properties characteristic of a Ca^{2+} sensor. *J Biol Chem* **277**, 16662-16672
 37. Saimi, Y., and Kung, C. (2002) Calmodulin as an ion channel subunit. *Annu Rev Physiol* **64**, 289-311
 38. Hackney, C. M., Mahendrasingam, S., Penn, A., and Fettiplace, R. (2005) The concentrations of calcium buffering proteins in mammalian cochlear hair cells. *J Neurosci* **25**, 7867-7875
 39. Schwaller, B., Celio, M. R., and Doglioni, C. (2004) Identification of calretinin and the alternatively spliced form calretinin-22k in primary pleural mesotheliomas and in their metastases. *Anticancer Res* **24**, 4003-4009
 40. Liang, H., DeMaria, C. D., Erickson, M. G., Mori, M. X., Alseikhan, B., and Yue, D. T. (2003) Unified mechanisms of Ca^{2+} regulation across the Ca^{2+} channel family. *Neuron* **39**, 951-960
 41. Zamponi, G. W. (2003) Regulation of presynaptic calcium channels by synaptic proteins. *J Pharmacol Sci* **92**, 79-83
 42. Andressen, C., Blumcke, I., and Celio, M. R. (1993) Calcium-binding proteins: selective markers of nerve cells. *Cell Tissue Res* **271**, 181-208
 43. Schwaller, B. (2007) Emerging Functions of the "Ca²⁺ Buffers" Parvalbumin, Calbindin D-28k and Calretinin in the Brain. in *Handbook of Neurochemistry and Molecular Neurobiology. Neural Protein Metabolism and Function* (Lajtha, A., Banik, N. ed.), Springer, New York. pp 197-222
 44. Christel, C., and Lee, A. (2012) Ca^{2+} -dependent modulation of voltage-gated Ca^{2+} channels. *Biochim Biophys Acta* **1820**, 1243-1252
 45. Lee, A., Zhou, H., Scheuer, T., and Catterall, W. A. (2003) Molecular determinants of Ca^{2+} /calmodulin-dependent regulation of $\text{Ca}_v2.1$ channels. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 16059-16064
 46. Chaudhuri, D., Issa, J. B., and Yue, D. T. (2007) Elementary mechanisms producing facilitation of $\text{Ca}_v2.1$ (P/Q-type) channels. *J Gen Physiol* **129**, 385-401
 47. Edmonds, B., Reyes, R., Schwaller, B., and Roberts, W. M. (2000) Calretinin modifies presynaptic calcium signaling in frog saccular hair cells. *Nature neuroscience* **3**, 786-790
 48. Findeisen, F., and Minor, D. L., Jr. (2009) Disruption of the IS6-AID linker affects voltage-gated calcium channel inactivation and facilitation. *J Gen Physiol* **133**, 327-343

49. Mochida, S., Sheng, Z. H., Baker, C., Kobayashi, H., and Catterall, W. A. (1996) Inhibition of neurotransmission by peptides containing the synaptic protein interaction site of N-type Ca^{2+} channels. *Neuron* **17**, 781-788
50. Watanabe, H., Yamashita, T., Saitoh, N., Kiyonaka, S., Iwamatsu, A., Campbell, K. P., Mori, Y., and Takahashi, T. (2010) Involvement of Ca^{2+} channel synprint site in synaptic vesicle endocytosis. *J Neurosci* **30**, 655-660
51. Rajapaksha, W. R., Wang, D., Davies, J. N., Chen, L., Zamponi, G. W., and Fisher, T. E. (2008) Novel splice variants of rat $\text{Ca}_v2.1$ that lack much of the synaptic protein interaction site are expressed in neuroendocrine cells. *J Biol Chem* **283**, 15997-16003
52. Inchauspe, C. G., Martini, F. J., Forsythe, I. D., and Uchitel, O. D. (2004) Functional compensation of P/Q by N-type channels blocks short-term plasticity at the calyx of held presynaptic terminal. *J Neurosci* **24**, 10379-10383
53. Felmy, F., and Schneggenburger, R. (2004) Developmental expression of the Ca^{2+} -binding proteins calretinin and parvalbumin at the calyx of held of rats and mice. *Eur J Neurosci* **20**, 1473-1482
54. Lin, K. H., Erazo-Fischer, E., and Taschenberger, H. (2012) Similar intracellular Ca^{2+} requirements for inactivation and facilitation of voltage-gated Ca^{2+} channels in a glutamatergic mammalian nerve terminal. *J Neurosci* **32**, 1261-1272
55. Lorteije, J. A., Rusu, S. I., Kushmerick, C., and Borst, J. G. (2009) Reliability and precision of the mouse calyx of Held synapse. *J Neurosci* **29**, 13770-13784
56. Dino, M. R., Willard, F. H., and Mugnaini, E. (1999) Distribution of unipolar brush cells and other calretinin immunoreactive components in the mammalian cerebellar cortex. *J Neurocytol* **28**, 99-123
57. Marini, A. M., Strauss, K. I., and Jacobowitz, D. M. (1997) Calretinin-containing neurons in rat cerebellar granule cell cultures. *Brain Res Bull* **42**, 279-288
58. Bearzatto, B., Servais, L., Roussel, C., Gall, D., Baba-Aissa, F., Schurmans, S., de Kerchove d'Exaerde, A., Cheron, G., and Schiffmann, S. N. (2006) Targeted calretinin expression in granule cells of calretinin-null mice restores normal cerebellar functions. *Faseb J* **20**, 380-382
59. Kreitzer, A. C., and Regehr, W. G. (2000) Modulation of transmission during trains at a cerebellar synapse. *J Neurosci* **20**, 1348-1357

FOOTNOTES

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FIGURE LEGENDS

Figure 1. CR binds to $\alpha_12.1$. (A) *Top*, Schematic of calretinin (CR) with functional (EF1-5, white) and non-functional (EF6, grey) Ca^{2+} -binding domains indicated. *Bottom*, consensus sequence for CR binding. (B) Schematic of $\text{Ca}_v2.1$ subunit, $\alpha_12.1$, with CR-binding domains (CRBs) in the II-III linker indicated (bold, underlined). Italics mark the synprint region. The sequence present in the GST- $\alpha_12.1$ fusion protein used for binding assays is shaded grey. (C) Pull-down assay of GFP-CR and GST-CRB1-3. GST (lanes 3,6) or GST-CRB1-3 (lanes 2,4) were immobilized on glutathione agarose beads and incubated with GFP-CR transfected HEK293T cell lysate in the presence of 1.6 mM CaCl_2 or 1 mM EGTA. (In) represents ~10% of the lysate used in the assay. *Top*, Western blot

with GFP antibody. *Bottom*, Integrity and levels of GST-fusion proteins were shown by Ponceau S staining. (D) *In vitro* assay for CR binding to GST- CRB1-3. GST or GST-CRB1-3 was tested for binding to Alexa 488-labeled CR in the absence (-) or presence (+) of Ca^{2+} (~300 μM). Fluorescence values were normalized to that obtained with 20 μM CR in the presence of Ca^{2+} (% Maximal binding). Data are from 3 independent experiments done in duplicate. (E) Coimmunoprecipitation of $\text{Ca}_v2.1$ and CR from HEK293T cells. Cells transfected with $\text{Ca}_v2.1$ subunits alone or cotransfected with GFP-CR were subject to lysis and immunoprecipitation with rabbit CR antibodies. Immunoprecipitated proteins were detected by Western blotting with $\alpha_{1.2.1}$ (top) or GFP (bottom) antibodies. (F) Coimmunoprecipitation of $\text{Ca}_v2.1$ and CR from mouse cerebellum. Mouse cerebellum lysates were incubated with rabbit antibodies against CR or control rabbit IgG. Immunoprecipitated proteins were detected by Western blotting with $\alpha_{1.2.1}$ (top) or calretinin (bottom) antibodies. Results shown in C and F are representative of 3 independent experiments; E is representative of 2 independent experiments.

Figure 2: CR modestly inhibits voltage-dependent activation of $\text{Ca}_v2.1$ I_{Ca} . Voltage protocol for current-voltage (I-V) relations and representative I_{Ca} (A) and I_{Ba} (B) current traces are shown for cells transfected with $\text{Ca}_v2.1$ alone or cotransfected with CR (+CR) with I-V curves shown below.

Figure 3: CR inhibits CDI and enhances $\text{Ca}_v2.1$ CDF. (A) Voltage protocol and representative traces for I_{Ca} and I_{Ba} are shown. P1 (black) and P2 (grey) test currents are overlaid for comparison. (B) The ratio of the amplitude of the P2 and P1 current (P2/P1) was plotted against prepulse voltage for I_{Ca} and I_{Ba} for cells transfected with $\text{Ca}_v2.1$ alone or $\text{Ca}_v2.1$ +CR. (C) *Left*, P2/P1 obtained with 0-mV prepulse voltage for $\text{Ca}_v2.1$ and $\text{Ca}_v2.1$ +CR. #, $p < 0.001$ compared to I_{Ca} ; * $p < 0.001$ by t-test. *Right*, CDI or CDF obtained with 0-mV prepulse represents P2/P1 ratio for I_{Ca} minus the mean P2/P1 ratio for I_{Ba} . * $p < 0.001$, t-test.

Figure 4: CR enhances $\text{Ca}_v2.1$ CDF during AP trains. (A) AP waveform and representative I_{Ca} evoked by 200-Hz train. Dashed line indicates initial current amplitude. Representative traces from the first (black) and 100th (grey) AP were overlaid for comparison for I_{Ca} and I_{Ba} in cells transfected with $\text{Ca}_v2.1$ alone or $\text{Ca}_v2.1$ +CR. (B) Fractional current represents test current amplitudes normalized to the first in the train and plotted against time. (C) The maximal fractional current at 0.5 s was compared for I_{Ca} and I_{Ba} . # $p < 0.001$ compared to I_{Ca} ; * $p < 0.03$ by t-test.

Figure 5: $\text{Ca}_v2.1$ channels lacking the CRBs are insensitive to CR modulation of CDI. (A-C) Same as in Fig. 3 except for $\text{Ca}_v2.1-1.2$ alone and $\text{Ca}_v2.1-1.2$ +CR.

Figure 6: $\text{Ca}_v2.1$ channels lacking the CRBs are insensitive to CR modulation of CDF. (A,B) Same as in Fig.4A,B except for $\text{Ca}_v2.1-1.2$ alone and $\text{Ca}_v2.1-1.2$ +CR. (C) CDF shown for wild-type $\text{Ca}_v2.1$ or $\text{Ca}_v2.1-1.2 \pm \text{CR}$. * $p < 0.005$.

Figure 7: A CR- binding peptide prevents effect of CR on $\text{Ca}_v2.1$ CDI and CDF. Same as Fig.3A,B and 4A,B but for I_{Ca} only and for cells transfected with $\text{Ca}_v2.1$ +CR or +CR+CRB1-3 (A,B) or $\text{Ca}_v2.1$ alone or +CRB1-3 (C,D).

Figure 1

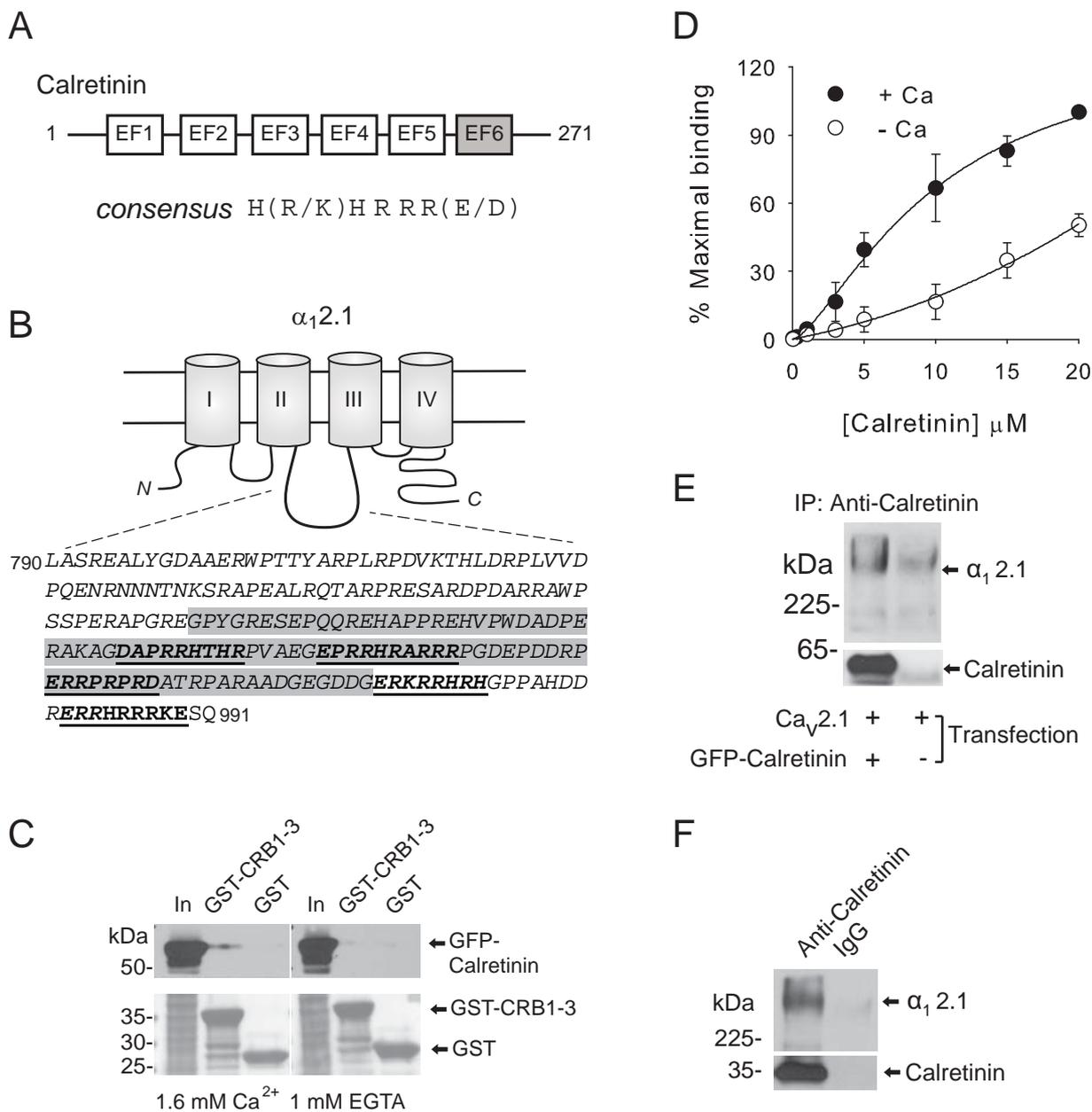


Figure 2

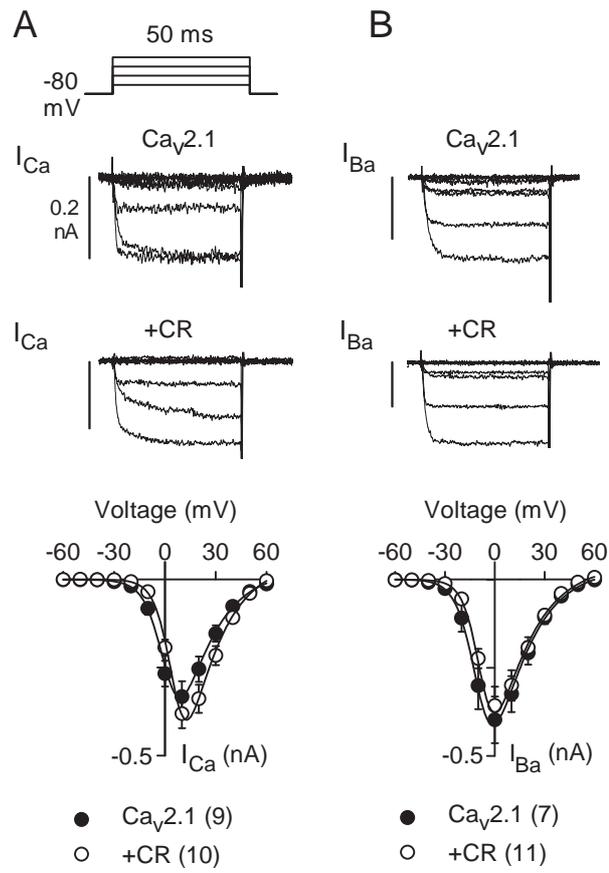


Figure 3

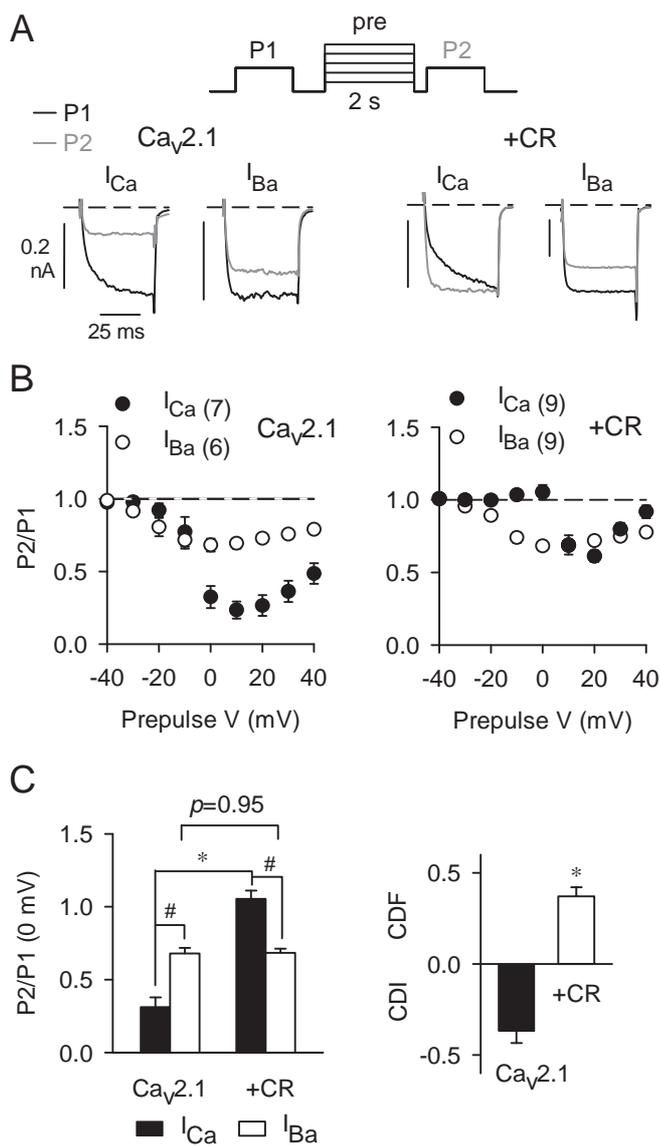


Figure 4

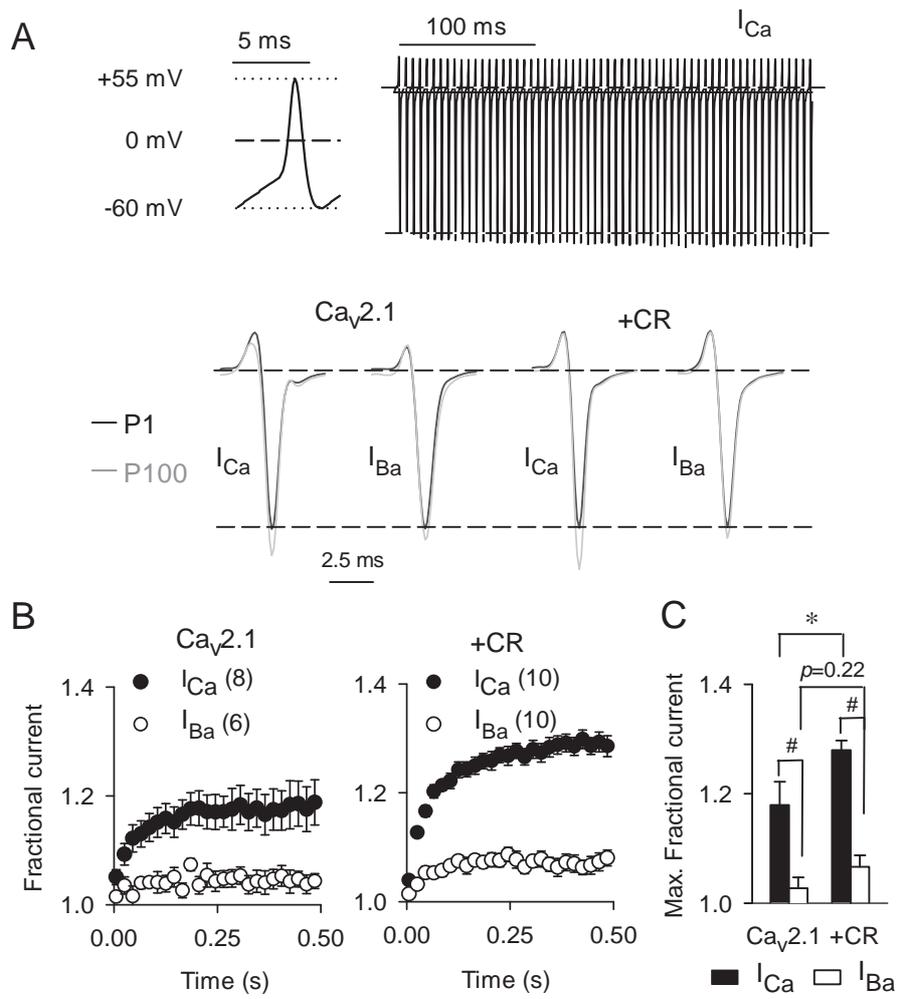


Figure 5

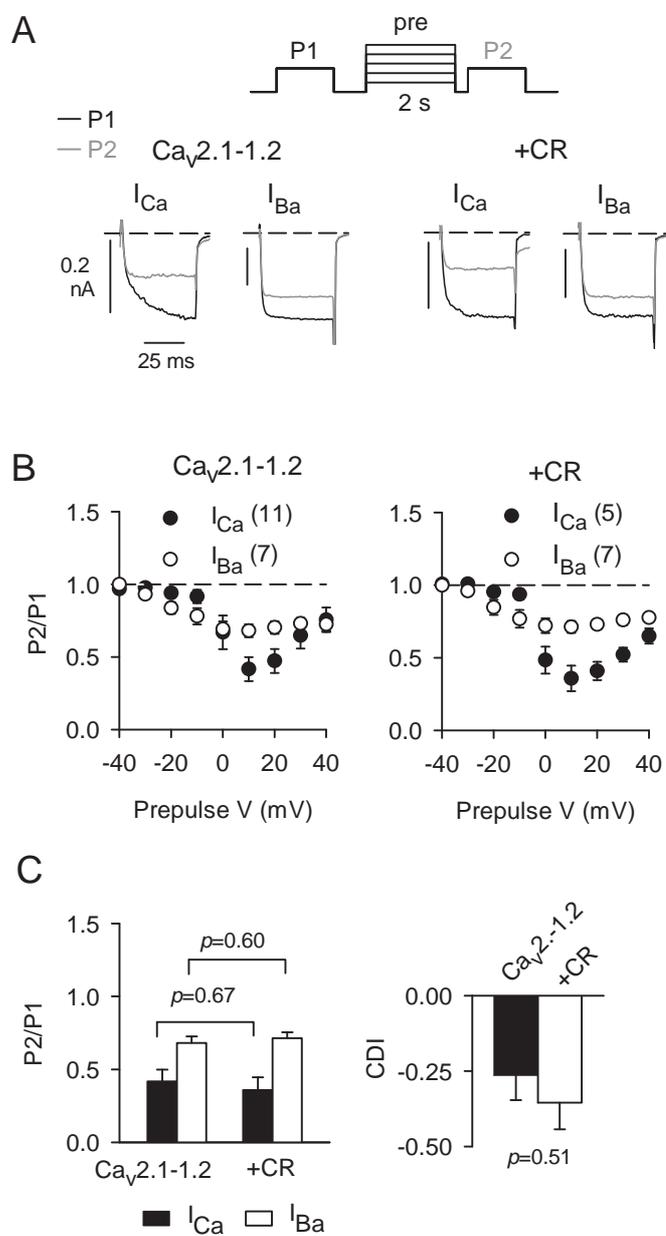


Figure 6

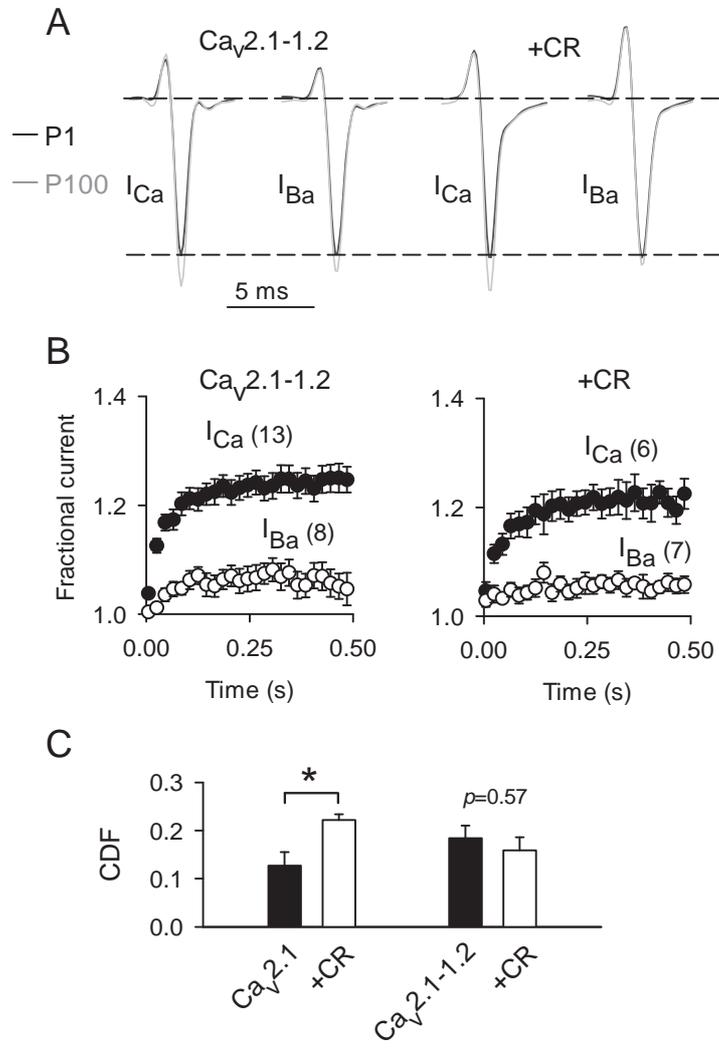


Figure 7

