Calretinin regulates Ca$^{2+}$-dependent inactivation and facilitation of Ca$_{v2.1}$ Ca$^{2+}$ channels through a direct interaction with the $\alpha_2$1 subunit

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Running title: Calretinin modulates Ca$_{v2.1}$ Ca$^{2+}$ channels

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Background: Ca$^{2+}$-dependent inactivation and facilitation of Ca$_{v2.1}$ Ca$^{2+}$ channels are major determinants of neuronal excitability and synaptic plasticity.

Result: The Ca$^{2+}$-binding protein calretinin interacts with Ca$_{v2.1}$ and inhibits Ca$^{2+}$-dependent inactivation and enhances facilitation of Ca$_{v2.1}$.

Conclusion: In addition to its role as a diffusible Ca$^{2+}$ buffer, calretinin can interact with targets such as Ca$_{v2.1}$ and modulate their function.

Significance: Calretinin-Ca$_{v2.1}$ interactions may shape Ca$^{2+}$ signaling dynamics in neurons.

SUMMARY: Voltage-gated 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_2$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_2$1. In pull-down assays, CR binding to fusion proteins containing these CRBs was largely Ca$^{2+}$-dependent. $\alpha_2$1 coimmunoprecipitated with CR antibodies from transfected cells and mouse cerebellum, which confirmed the existence of CR-Ca$_{v2.1}$ complexes in vitro and in vivo. In HEK293T cells, CR significantly decreased Ca$_{v2.1}$ CDI and increased CDF. CR binding to $\alpha_2$1 was required for these effects, since they were not observed upon substitution of the II-III linker of $\alpha_2$1 with that from the Ca$_{v1.2}$ $\alpha_1$ subunit ($\alpha_1$1.2) 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_1$2.1. Our findings about calretinin-Ca$_{v2.1}$ interactions may shed light on the regulation of Ca$^{2+}$ signaling in neurons.
highlight an unexpected role for CR in directly modulating effectors such as Ca,2.1, which may have major consequences for Ca\(^{2+}\)-signaling and neuronal excitability.

Voltage-gated Ca,2.1 channels mediate Ca\(^{2+}\) 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 Ca\(^{2+}\) concentration (2,3), modulation of Ca,2.1 properties can greatly alter synaptic output and contribute to mechanisms of synaptic plasticity (4).

Like other Ca\(_v\) channels, Ca,2.1 channels undergo a prominent feedback regulation by incoming Ca\(^{2+}\) ions, which depends on calmodulin (CaM) (5,6). However, unlike other Ca\(_v\) channels, CaM binding to the cytoplasmic C-terminal domain of \(\alpha_1\) mediates both a negative and positive regulation by incoming Ca\(^{2+}\) ions, Ca\(^{2+}\)-dependent inactivation (CDI) and facilitation (CDF) (7). Both CDI and CDF have been described for presynaptic Ca,2.1 channels at central synapses (8-10) and can cause short-term depression and facilitation, respectively, of neurotransmitter release (11).

Ca\(^{2+}\)-dependent modulation of native Ca,2.1 channels is heterogeneous in neurons (12), which can be due to alternative splicing of sequences encoding the \(\alpha_1\) C-terminal domain (13,14) or differences in auxiliary Ca,\(\beta\) subunit expression (15). In addition, neuron-specific Ca\(^{2+}\) binding proteins related to CaM can influence the extent of Ca,2.1 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 \(\alpha_1\), but structural differences from CaM underly their distinct modulation of Ca,2.1 (17,18).

Within the EF-hand superfamily of Ca\(^{2+}\) binding proteins, CaM, CaBP1, and VILIP-2 are considered Ca\(^{2+}\) sensors, so defined by their abilities to undergo Ca\(^{2+}\)-dependent conformational change and interact with effectors. In contrast, Ca\(^{2+}\) binding proteins such as parvalbumin and calbindin D-28k generally act as diffusible Ca\(^{2+}\) buffers that modulate cytoplasmic Ca\(^{2+}\) signals (19). Like Ca\(^{2+}\) chelation by EGTA (7), parvalbumin and calbindin D-28k can significantly alter CDI of Ca,2.1 channels in transfected HEK293T cells (20). However, as parvalbumin and calbindin D-28k were not reported to associate directly with \(\alpha_1\), their actions on Ca,2.1 CDI are likely to be indirect and due primarily to their actions as diffusible Ca\(^{2+}\) buffers (20).

Calretinin (CR) is a EF-hand Ca\(^{2+}\)-binding protein that is highly expressed in cerebellar granule neurons where Ca,2.1 channels are also expressed (19,21,22). Like parvalbumin and calbindin D-28k, CR is considered mainly as a Ca\(^{2+}\) signal modulator in neurons. Based on its Ca\(^{2+}\) chelating properties, CR can significantly shape the spatiotemporal properties of Ca\(^{2+}\) signals generated by plasma membrane and intracellular Ca\(^{2+}\) channels (23). Yet, CR undergoes large conformational changes upon binding to Ca\(^{2+}\) (24,25), which may mediate Ca\(^{2+}\)-dependent interactions with integral membrane proteins (26). While investigating the potential targets of CR, we discovered a consensus CR-binding motif in \(\alpha_1\). Ca\(^{2+}\)-dependent binding of CR to this region nullifies CDI and enhances CDF in transfected HEK293T cells, which suggests that local tethering of CR to \(\alpha_1\) is required for channel modulation. Our findings provide the first evidence that CR can directly alter Ca\(^{2+}\) 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,2.1 subunit cDNAs were used: \(\alpha_1\) (rbA isofrom (29)), \(\beta_2\) (30), and \(\alpha_2\delta\) (31). GST-\(\alpha_1\) 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 Ca\(^{2+}\) channel \(\alpha_{1.1-1.2}\) subunit, amino acids 864 – 983 from rat brain \(\alpha_{1.2}\) were replaced by amino acids 807 – 923 of rat brain \(\alpha_{1.2}\) (rbCII, (32)). The corresponding DNA sequence of \(\alpha_{1.2}\) was amplified by PCR and cloned into Ascl/SgrAI sites of the \(\alpha_{2.1}\)-containing expression plasmid. GFP-CR was generated by cloning a PCR fragment containing the cDNA coding for human CR (836 bp) in the plasmid pEFGP-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 \(\alpha_{2.1}\) (mcherry-CRB1-3), the corresponding sequence (aa 899-953) and Kozak sequence was amplified from rat brain \(\alpha_{2.1}\) by PCR and subcloned into XhoI and HindIII restriction sites of pEFGFPN1-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% CO\(_2\). 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\(_{2.1}\) subunit cDNAs: \(\alpha_{2.1}\) (2 \(\mu\)g), \(\beta_{2A}\) (1 \(\mu\)g), and \(\alpha_{2}\delta\) (1 \(\mu\)g). pEFGFPN1 or GFP-CR was cotransfected (1 \(\mu\)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 \(\mu\)g). For coimmunoprecipitation experiments, cells were plated in 60 mm dishes and transfected with Ca\(_{2.1}\) subunits GFP-\(\alpha_{2.1}\) (3.2 \(\mu\)g), \(\beta_{2A}\) (1.6 \(\mu\)g), and \(\alpha_{2}\delta\) (1.6 \(\mu\)g) and pEFGFPN1 (1.6 \(\mu\)g) or GFP-CR (1.6 \(\mu\)g).

**Phage display and binding assays**

Purified human recombinant CR-coated 7.5 cm\(^2\) plastic plates and the Ph.D.\(^{TM}\)-7 Phage Display Peptide Library Kit (New England Biolabs Inc, Biocencept, Allschwil, Switerzerland) 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-\(\alpha_{2.1}\) 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 \(\mu\)M) was incubated with GST - \(\alpha_{2.1}\) fusion proteins or GST control (10 \(\mu\)M) in binding buffer (in mM: 140 K-glutamate, 2 ATP, 1.25 MgCl\(_2\), 20 PIPES, 0.4 EGTA; pH 7.2, adjusted with KOH). Ca\(^{2+}\)-containing solutions included 1.6 mM CaCl\(_2\) (Ca\(^{2+}\):~300 \(\mu\)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-\(\alpha_{2.1}\) 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 CaCl\(_2\) 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-Į12.1 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-Į12.1 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,2.1
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 α,2.1 (Fig.1B). The CRBs are C-terminal to the synaptic protein-interaction site
(“synprint”; 33) and are highly conserved in α1.2.1 between species. The CRB-containing region is also present in the Ca,2.2 α1 subunit (α1.2.2, Supplementary Fig.1), but absent in Ca,1 α1 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 α1.2.1 (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 α1.2.1, GST-CRB1-3 was incubated with varying amounts of fluorescently tagged CR. Binding was relatively low-affinity (EC$_{50}$=10 μM) 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 ± 7.9% and 32.6 ± 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 α1.2.1.

If CR is an interacting partner of Ca,2.1, it should also associate with the intact channel. To test this prediction, we coexpressed CR and Ca,2.1 in HEK293T cells and used CR antibodies in coimmunoprecipitation experiments. CR antibodies brought down α1.2.1 in cells cotransfected with Ca,2.1+ CR. Some α1.2.1 was brought down non-specifically by CR antibodies since a small amount of α1.2.1 was detected in immunoprecipitations from cells transfected with Ca,2.1 alone (Fig.1E). However, a larger signal corresponding to α1.2.1 was consistently detected when Ca,2.1 was coexpressed with CR, indicating some specific association of CR with α1.2.1. To verify these results in the native context, we performed coimmunoprecipitation experiments with extracts from mouse cerebellum given that CR and Ca,2.1 are both expressed highly in cerebellar granule neurons (19,21,22). In these experiments, CR antibodies but not control IgG coimmunoprecipitated α1.2.1 (Fig.1F). Together, these results demonstrate that CR associates with recombinant and native Ca,2.1 channels in the brain.

**CR modulates CDI and CDF of Ca,2.1**

To investigate the functional consequences of CR interacting with α1.2.1, we compared the properties of Ca,2.1 transfected alone or cotransfected with CR in HEK293T cells. Because Ca,2.1 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 $I_{Ca}$ ($V_{1/2} = 2.7$ mV±0.7 mV for Ca,2.1 alone vs. $7.3±0.4$ mV for Ca,2.1+CR, $p<0.001$; $k$ = -5.4 ±0.2 for Ca,2.1 alone vs. -4.8±0.1 for Ca,2.1+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 Ca,2.1. 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 Ca,2.1. 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 Ca,2.1, $I_{Ca}$, 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 \( \mathrm{Ca}_{2.1} \) 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 \( \mathrm{Ca}_{2.1} \) 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 \( \mathrm{Ca}_{2.1} \) alone (\( \tau_{P1}=6 \pm 1.3 \) ms vs. \( \tau_{P2}=2 \pm 0.3 \) ms, \( n=6 \), \( p=0.02 \)) and \( \mathrm{Ca}_{2.1}+\text{CR} \) (\( \tau_{P1}=12.4 \pm 1 \) 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 \( \mathrm{Ca}_{2.1} \) alone (~125%, \( p=0.001 \)). To quantitate the \( \mathrm{Ca}^{2+}\)-dependent modulation seen in activation kinetics of the \( P2 \) compared to the \( P1 \) current for both \( \mathrm{Ca}_{2.1} \) alone (\( \tau_{P1}=6 \pm 1.3 \) ms vs. \( \tau_{P2}=2 \pm 0.3 \) ms, \( n=6 \), \( p=0.02 \)) and \( \mathrm{Ca}_{2.1}+\text{CR} \) (\( \tau_{P1}=12.4 \pm 1 \) 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 \( \mathrm{Ca}_{2.1} \) alone (~125%, \( p=0.001 \)).

We further analyzed the effects of CR on \( \mathrm{Ca}_{2.1} \) 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 \( \mathrm{Ca}_{2.1} \) was significantly greater with CR (\( 0.22 \pm 0.01 \), \( n=10 \)) than without (\( 0.13 \pm 0.03 \), \( n=8 \), \( p<0.01 \), t-test). These results confirm that CR enhances CDF of \( \mathrm{Ca}_{2.1} \).

These effects of CR on \( \mathrm{Ca}_{2.1} \) CDI and CDF could be due to its actions as a freely diffusible \( \mathrm{Ca}^{2+} \) buffer. Alternatively, CR binding to the \( \alpha_{2.1} \) 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 \( \alpha_{2.1} \) are not conserved in the \( \mathrm{Ca}_{1.2} \) \( \alpha_1 \) subunit (\( \alpha_{1.2} \)). If CR binding to \( \alpha_{2.1} \) is necessary for \( \mathrm{Ca}_{2.1} \) modulation, then chimeric channels in which the 2-3 linker of \( \alpha_{1.2} \) is substituted for that in \( \alpha_{2.1} \) (\( \mathrm{Ca}_{2.1}-1.2 \)), should prevent effects of CR on CDI and CDF. Consistent with this prediction, \( \mathrm{Ca}_{2.1}-1.2 \) 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 \( \mathrm{Ca}_{2.1}-1.2 \) alone and cells cotransfected with CR (\( p=0.57 \); Fig.6). These results show that the CRBs are required for CR modulation of \( \mathrm{Ca}_{2.1} \) CDI and CDF.

To verify the importance of CR binding to the CRBs for \( \mathrm{Ca}_{2.1} \) modulation, we tested the impact of a peptide which should competitively displace CR from binding sites in the \( \alpha_{2.1} \) 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 \( \mathrm{Ca}_{2.1}+\text{CR} \), CRB1-3 significantly opposed the decrease in CDI caused by CR (for a 0-mV prepulse, \( P2/P1=1.0 \pm 0.05 \) for \( \mathrm{Ca}_{2.1}+\text{CR} \) vs. \( 0.58 \pm 0.11 \) 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 \( \mathrm{Ca}_{2.1} \).
alone and those cotransfected with Ca$_{\text{a},2.1}$+CRB1-3 (Fig.7C,D), which argued against non-specific inhibitory effects of CRB1-3 on Ca$_{\text{a},2.1}$ that were independent of CR modulation. Taken together, our results support a mechanism in which CR binding to the $\alpha_{\text{a},2.1}$ II-III linker directly inhibits Ca$_{\text{a},2.1}$ CDI and enables enhanced CDF of Ca$_{\text{a},2.1}$ during repetitive stimuli.

**DISCUSSION**

Our study provides key evidence for a new role for CR as an integral component of Ca$_{\text{a},2.1}$ complexes that modulates Ca$_{\text{a},2.1}$ function. First, CR binds in a Ca$^{2+}$-dependent manner to basic motifs in the $\alpha_{\text{a},2.1}$ II-III linker. Second, CR forms a complex with Ca$_{\text{a},2.1}$ channels in the brain. Third, the interaction of CR with the $\alpha_{\text{a},2.1}$ II-III linker inhibits CDI and enhances CDF of Ca$_{\text{a},2.1}$ channels and enhances CDF in transfected HEK293T cells. Because of the cellular overlap in CR and Ca$_{\text{a},2.1}$ expression in the brain (22,34), CR interactions with Ca$_{\text{a},2.1}$ should be considered in models of how this protein controls Ca$^{2+}$ signaling and excitability in neurons.

**Ca$^{2+}$ dependent binding of CR to effectors**

CR has long been considered a diffusible Ca$^{2+}$ buffer which can alter spatiotemporal aspects of Ca$^{2+}$ 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 “Ca$^{2+}$ sensors” such as CaM. First, CR undergoes large conformational changes upon binding to Ca$^{2+}$ (24,25). Similar Ca$^{2+}$-dependent structural changes have been reported for the related EF-hand proteins, calbindin D28k and secretagogin (35,36). As in CaM, Ca$^{2+}$ binding to CR may expose hydrophobic regions of the protein, which allow Ca$^{2+}$-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 Ca$^{2+}$ (26). These latter findings could be explained by Ca$^{2+}$-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 $\mu$M) of CR for the $\alpha_{\text{a},2.1}$ CRB sequences in GST-CRB1-3 (Fig.1D), we believe this may underestimate the ability of CR to interact with $\alpha_{\text{a},2.1}$ 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 $\mu$M (27,38). These concentrations of CR would be sufficient to saturate the CRB sequence(s) in $\alpha_{\text{a},2.1}$ 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 $\alpha_{\text{a},2.1}$ 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 $\alpha_{\text{a},2.1}$ 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 $\alpha_{\text{a},2.1}$.

While our findings that CR coimmunoprecipitates with Ca$_{\text{a},2.1}$ channels in the brain (Fig.1F) clearly implicate Ca$_{\text{a},2.1}$ as a CR target, it is important to note that basic amino acid sequences similar to the $\alpha_{\text{a},2.1}$ CRBs are likely present in other effectors. In particular, the CRBs are conserved in the “synprint” region of the Ca$_{\text{a},2.2}$ $\alpha_{\text{I}}$ subunit ($\alpha_{\text{a},2.2}$, Supp.Fig.1). While Ca$_{\text{a},2.2}$ channels do
not undergo CDF (40), synaptic protein interactions with the II-III linker of α_{2.2} regulate voltage-dependent inactivation of Ca_{2.2} and inhibition of these channels by heterotrimeric G-proteins (41). Thus, it is possible that CR binding to the CRBs in α_{2.2} could have important consequences for the modulation of Ca_{2.2} channels in neurons. The characterization of other CR-interacting proteins other than Ca_{2.1}, 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_{2.1} CDI/CDF modulation by CR**

Significant progress has been made in elucidating the mechanisms underlying Ca_{2.1} 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 Ca_{2.1} 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 Ca_{2.1} inhibited CDI. The remarkable result is that CR accomplishes this through its association with the α_{2.1} II-III linker (Figs. 6,7). Our biochemical and electrophysiological experiments show that disabling CR interactions with the α_{2.1} 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 Ca_{2} α_{1} subunits, auxiliary Ca_{αβ} subunit interactions with the I-II linker have been shown to modulate CDI and CDF of Ca_{1.2} 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 α_{2.1} II-III linker overlap with the bipartite synprint site in α_{2.1}. Interactions between SNAREs and the synprint are thought to promote efficient coupling of Ca_{2} 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 Ca_{2.1} 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 Ca_{2.1} Ca^{2+} signaling between neuronal sub-types.

**Neurophysiological significance of CR/Ca_{2.1}**

Immunohistochemical analyses indicate a number of neuronal cell groups in which CR and Ca_{2.1} colocalize. Ca_{2.1} 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 Ca_{2.1} 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 Ca\(_{2.1}\) with CR from mouse cerebellum would indicate, CR/Ca\(_{2.1}\) 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 \textit{in vivo} \(^{(27,28,58)}\). With respect to Ca\(_{2.1}\) in granule cells, loss of CR should inhibit Ca\(_{2.1}\) Ca\(^{2+}\) influx by enhancing CDI (Fig.3). Decreased I\(_{Ca}\) may seem at odds with the hyperexcitable phenotype of 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 Ca\(_{2.1}\) 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 CR\(^{-/-}\) granule cells. In addition, Ca\(_{2.1}/CR\) 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 Ca\(_{2.1}\) 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


**FOOTNOTES**

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**FIGURE LEGENDS**

Figure 1. CR binds to \( \alpha_{1} \)2.1. (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 Ca\(_{v} \)2.1 subunit, \( \alpha_{1} \)2.1, with CR-binding domains (CRBs) in the II-III linker indicated (bold, underlined). Italics mark the synprint region. The sequence present in the GST- \( \alpha_{1} \)2.1 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 Ca$_{2.1}$ and CR from HEK293T cells. Cells transfected with Ca$_{2.1}$ 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 α$_{2.1}$ (top) or GFP (bottom) antibodies. (F) Coimmunoprecipitation of Ca$_{2.1}$ 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 α$_{2.1}$ (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 Ca$_{2.1}$ $I_{\text{Ca}}$. Voltage protocol for current-voltage (I-V) relations and representative $I_{\text{Ca}}$ (A) and $I_{\text{Ba}}$ (B) current traces are shown for cells transfected with Ca$_{2.1}$ alone or cotransfected with CR (+CR) with I-V curves shown below.

Figure 3: CR inhibits CDI and enhances Ca$_{2.1}$ CDF. (A) Voltage protocol and representative traces for $I_{\text{Ca}}$ and $I_{\text{Ba}}$ are shown. P1 (black) and P2 (grey) test currents are overlayed for comparison. (B) The ratio of the amplitude of the P2 and P1 current (P2/P1) was plotted against prepulse voltage for $I_{\text{Ca}}$ and $I_{\text{Ba}}$ for cells transfected with Ca$_{2.1}$ alone or Ca$_{2.1}$+CR. (C) Left, P2/P1 obtained with 0-mV prepulse voltage for Ca$_{2.1}$ and Ca$_{2.1}$+CR. #, $p<0.001$ compared to $I_{\text{Ca}}$; *$p<0.001$ by t-test. Right, CDI or CDF obtained with 0-mV prepulse represents P2/P1 ratio for $I_{\text{Ca}}$ minus the mean P2/P1 ratio for $I_{\text{Ba}}$. *$p<0.001$, t-test.

Figure 4: CR enhances Ca$_{2.1}$ CDF during AP trains. (A) AP waveform and representative $I_{\text{Ca}}$ 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 $I_{\text{Ca}}$ and $I_{\text{Ba}}$ in cells transfected with Ca$_{2.1}$ alone or Ca$_{2.1}$+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 $I_{\text{Ca}}$ and $I_{\text{Ba}}$. #p<0.001 compared to $I_{\text{Ca}}$; *p<0.03 by t-test.

Figure 5: Ca$_{2.1}$ channels lacking the CRBs are insensitive to CR modulation of CDI. (A-C) Same as in Fig. 3 except for Ca$_{2.1}$-1.2 alone and Ca$_{2.1}$-1.2+CR.

Figure 6: Ca$_{2.1}$ channels lacking the CRBs are insensitive to CR modulation of CDF. (A,B) Same as in Fig.4A,B except for Ca$_{2.1}$-1.2 alone and Ca$_{2.1}$-1.2+CR. (C) CDF shown for wild-type Ca$_{2.1}$ or Ca$_{2.1}$-1.2 ± CR. *p<0.005.

Figure 7: A CR-binding peptide prevents effect of CR on Ca$_{2.1}$ CDI and CDF. Same as Fig.3A,B and 4A,B but for $I_{\text{Ca}}$ only and for cells transfected with Ca$_{2.1}$+CR or +CR+CRB1-3 (A,B) or Ca$_{2.1}$ alone or +CRB1-3 (C,D).
Figure 1

A

Calretinin

1  EF1 - EF2 - EF3 - EF4 - EF5 - EF6 - 271

consensus  H(R/K) H R R (E/D)

B

α1.2.1

C

GST-CRB1-3  GST  In  GST-CRB1-3  GST  In

kDa

50- 35- 30- 25-

1.6 mM Ca2+  1 mM EGTA

GFP-Calretinin

GST-CRB1-3

GST

D

% Maximal binding

[Calretinin] μM

0  5  10  15  20

E

IP: Anti-Calretinin

kDa

225- 65-

Ca2+ + + - Transfection

F

Anti-Calretinin

kDa

225- 65-

α1.2.1

Calretinin
Figure 2

A

B

ICa (nA)

Cav2.1 (9)

+CR (10)

IBa (nA)

Cav2.1 (7)

+CR (11)

-60 -30 0 30 60

Voltage (mV)

50 ms

0.2 nA

-80 mV
Figure 3

A

B

C

* $p=0.95$
Figure 5

A

B

C
**Figure 6**

A. Time (s)

- P1
- P100

ICa ICa IBa IBa

5 ms

B. Fractionsal current

- CaV2.1-1.2 ICa (13) ICa (6)
- Ba (8) Ba (7)

C. Cav2.1-1.2 +CR

*p*=0.57

CDF

ICa xCR CaV2.1-1.2 xCR
Figure 7

A

- P1
- P2

+CR
+CR+CRB1-3

0.2 nA
25 ms

P2/P1

ICa

P2/P1

I_Ca

+CR (9)
+CR+CRB1-3 (8)

Prepulse V (mV)

-40 -20 0 20 40

B

+CR
+CR+CRB1-3

2.5 ms

P1
P200

Fractional current

0.0 0.5 1.0

Time (s)

0.0 0.5 1.0

Fractional current

0.0 0.5 1.0

0.0 0.5 1.0

C

- P1
- P2

Ca_{2.1}
+CRB1-3

0.2 nA
25 ms

D

Ca_{2.1}
+CRB1-3

2.5 ms

P1
P200

Fractional current

0.0 0.5 1.0

Time (s)

0.0 0.5 1.0

Fractional current

0.0 0.5 1.0

0.0 0.5 1.0

-40 -20 0 20 40