Endogenous and Exogenous Ca²⁺ Buffers Differentially Modulate Ca²⁺-dependent Inactivation of Caᵥ2.1 Ca²⁺ Channels*

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Lisa Kreiner and Amy Lee
From the Department of Pharmacology and Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, Georgia 30322

Voltage-gated Ca²⁺ channels undergo a negative feedback regulation by Ca²⁺ ions, Ca²⁺-dependent inactivation, which is important for restricting Ca²⁺ signals in nerve and muscle. Although the molecular details underlying Ca²⁺-dependent inactivation have been characterized, little is known about how this process might be modulated in excitable cells. Based on previous findings that Ca²⁺-dependent inactivation of Caᵥ2.1 (P/Q-type) Ca²⁺ channels is suppressed by strong cytoplasmic Ca²⁺ buffering, we investigated how factors that regulate cellular Ca²⁺ levels affect inactivation of Caᵥ2.1 Ca²⁺ currents in transfected 293T cells. We found that inactivation of Caᵥ2.1 Ca²⁺ currents increased exponentially with current amplitude in ways that were unexpected from behavior as passive Ca²⁺ buffers. We conclude that Ca²⁺-dependent inactivation of Caᵥ2.1 depends on a subplasmalemmal Ca²⁺ microdomain that is affected by the amplitude of the Ca²⁺ current and differentially modulated by distinct Ca²⁺ buffers. A fundamental distinction between CDF and CDI is their sensitivity to cytoplasmic Ca²⁺ buffering. The blockade of CDI, but not CDF, by high concentrations of the Ca²⁺ chelators EGTA and BAPTA (8, 12) suggests that the extent to which Caᵥ2.1 channels undergo CDI may largely be influenced by factors that regulate intracellular Ca²⁺ concentrations. Such factors include parvalbumin (PV) and calbindin (CB), which are EF-hand Ca²⁺-binding proteins that alter the amplitude and time course of Ca²⁺ signals in some nerve and muscle cells (13–17). Unlike calmodulin, which directly interacts with and confers Ca²⁺-dependent regulation to numerous effectors (18), PV and CB were generally thought to act as passive Ca²⁺ buffers, which help protect cells from Ca²⁺ overloads. However, by modifying the spatial and temporal aspects of intracellular Ca²⁺ elevations, PV and CB can influence Ca²⁺ signals that modulate the activity of inositol 1,4,5-trisphosphate receptors (19, 20). Similarly, PV and CB might physiologically regulate Ca²⁺-dependent modulation of Caᵥ2.1, as PV and CB are concentrated in subsets of neurons, such as cerebellar Purkinje neurons, where Caᵥ2.1 channels are also highly expressed (21–24). Previous studies implicate a role for PV and CB in modulating CDI of L-type voltage-gated Ca²⁺ channels in neurons (25, 26), although whether these proteins also affect Caᵥ2.1 (P/Q-type) channels is not known.

In this study, we compared the effects of Ca²⁺ buffers (EGTA and BAPTA) and Ca²⁺-buffering proteins (PV and CB) on CDI of Caᵥ2.1 in transfected 293T cells. Our analyses indicate that inactivation of Caᵥ2.1 Ca²⁺ currents varies significantly with current amplitude and is more sensitive to Ca²⁺ buffering by EGTA than BAPTA. PV and CB do not simply replicate the effects of EGTA and BAPTA, but differentially altered the current dependence of CDI. These findings reveal the importance of cellular Ca²⁺-buffering mechanisms in the negative feedback regulation of Caᵥ2.1 channels by Ca²⁺, which may further diversify the properties of these channels in different neuronal cell types (27).

EXPERIMENTAL PROCEDURES

cDNA Expression Constructs—Caᵥ2.1 subunits used in electrophysiological experiments were α₂.1 (rbA isoform), β₃.₆, and α₂δ (28–30). cDNAs corresponding to rat parvalbumin and calbindin were isolated by PCR amplification with specific primers from a rat brain cDNA library. Parvalbumin was subcloned into the HindIII/BamHI sites of pcDNA3.1+, and calbindin was subcloned into the BamHI/XhoI sites of pcDNA3.1-topo. The PVcdc5 mutant containing amino acid substitutions D51A, E62V, D90A, and E101V was based on that described by Pauls et al. (31) and generated by multiple rounds of QuikChange mutagenesis and subcloning into pcDNA3.1+. The identity of all cDNA constructs was confirmed by sequencing prior to use in electrophysiological experiments.

Cell Culture and Transfection—293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine

Caᵥ2.1 (P/Q-type) voltage-gated Ca²⁺ channels mediate Ca²⁺ signals that regulate neuronal excitability, synapse formation, and neurotransmitter release (1–5). Fidelity of Ca²⁺ signaling by Caᵥ2.1 requires fine control of voltage-gated Ca²⁺ entry, in part by the Ca²⁺ ions that permeate the channel. Self-regulation of Caᵥ2.1 channels by Ca²⁺ is manifest as an initial increase (facilitation) and gradual decrease (inactivation) in Ca²⁺ current amplitude during high frequency stimuli (6–8). Ca²⁺-dependent facilitation (CDF) and inactivation (CDI) of Caᵥ2.1 channels depend on calmodulin binding to the pore-forming α₁ subunit of Caᵥ2.1 (7, 9) and can cause activity-dependent changes in synaptic efficacy (6, 10, 11).

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1 To whom correspondence should be addressed: Dept. of Pharmacology, Emory University School of Medicine, 5123 Rollins Research Bldg., 1510 Clifton Rd., Atlanta, GA 30322. Tel.: 404-727-5991; Fax: 404-727-0365; E-mail: alee@pharm.emory.edu.

2 The abbreviations used are: CDF, Ca²⁺-dependent facilitation; CDI, Ca²⁺-dependent inactivation; PV, parvalbumin; CB, calbindin; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N’-tetraacetic acid; IP, inositol phosphate.
serum at 37 °C under 5% CO₂. Cells plated in 35-mm tissue culture dishes were grown to 65–80% confluency and transfected with GenePORTER transfection reagent (Gene Therapy Systems Inc., San Diego, CA) according to the manufacturer’s protocol with a 1:1 molar ratio of cDNAs for Ca²⁺ channel subunits (total of 5 μg) and 0.7 μg of a CD8 expression plasmid for identification of transfected cells. Parvalbumin and calbindin cDNAs were transfected at a 1:1 molar ratio with Ca²⁺ expression plasmids for identification of transfected cells. 

Western Blots—293T cells, plated and transfected as for electrophysiological experiments, were homogenized in ice-cold lysis buffer (25 mM Tris, pH 7.4), 137 mM NaCl, 2.7 mM KCl, 0.1% phenylmethylsulfonyl fluoride, and 1% Triton X-100) and stored at −20 °C until use. Cell lysates (50 μg) were electrophoresed on denaturing 4–20% Tris-glycine gels (Invitrogen) and transferred to a nitrocellulose membrane that was blocked in 3% milk/TBS and incubated with antibodies against PV (1:1000, Chemicon International, Temecula, CA), or CB (1:1000, Chemicon International). Chemiluminescent detection was achieved with horseradish peroxidase-conjugated secondary antibodies (1:2000, Amersham Biosciences) and ECL reagents (Amersham Biosciences).

Electrophysiological Recordings—At least 48 h after transfection, 293T cells were incubated with CD8 antibody-coated microspheres (Dynal, Oslo, Norway) for identification of transfected cells. Ca²⁺ or Ba²⁺ currents were recorded in whole cell patch clamp recordings with a HEKA EPC-9 patch-clamp amplifier driven by PULSE software (HEKA Electronics, Lambrecht/Pfalz, Germany). Leak and capacitive transients were subtracted using a P/I-4 protocol. Extracellular recording solutions contained (in mM): 150 Tris, 1 MgCl₂, and 10 CaCl₂ or 10 BaCl₂. Intracellular recording solutions contained (in mM): 130 N-methyl-d-glucamine, 60 HEPES, 1 MgCl₂, 2 Mg-ATP, and EGTA (0.5 mM) or BAPTA (0.5 mM or 10 mM). The pH of extracellular and intracellular recording solutions was adjusted to 7.3 with methanesulfonic acid. Because of shifts in the activation curve of −10 and +10 mV when extracellular Ba²⁺ or intracellular BAPTA were used, respectively, voltage protocols were adjusted to compensate for this difference as noted.

Data Analysis—All data were analyzed using custom written procedures in IGOR Pro software (Wavemetrics, Portland, OR). Averaged data represent the mean ± S.E. Statistical differences in averaged inactivation (I_{cell}/I_{peak}) between groups was determined by Student’s t test. I-V curves were fit with the function: g(V-E)/(1 + Exp([V-V_{1/2}]/k)) where g is the maximum conductance, V is the test potential, E is the apparent reversal potential, V_{1/2} is the potential of half-activation, k is the slope factor, and b is the baseline. Linear and nonlinear regression and statistical analyses were done with Sigma Plot (SPSS, Inc., Chicago, IL). Significant deviations of percent inactivation data from regression models were determined by Runs test. Data describing current dependence of I_{ca} inactivation were fit with a nonlinear regression equation, y = ax^b where y is % inactivation, x is the current amplitude, and a and b are constants. F-tests were used for comparisons of nonlinear regression curves, with statistical significance considered as p < 0.05.

RESULTS

Current Dependence of CDI and Sensitivity to EGTA—Ca²⁺ microdomains near the pore of individual Ca²⁺ channels may reach micromolar concentrations, but are too short-lived to be significantly buffered by high concentrations of slow Ca²⁺ buffers such as EGTA or low concentrations of fast Ca²⁺ buffers such as BAPTA (32). For this reason, the blockade of CDI of Ca²⁺/1 by high intracellular concentrations of EGTA and BAPTA (10 mM) implies a requirement for a “global” Ca²⁺ signal that is supported by multiple open channels (8, 9, 12). A simple prediction of this model is that CDI should increase to some extent with the amplitude of the whole cell Ca²⁺ current (I_{ca}). In support of this prediction, human splice variants of α2.1 with poor expression levels in transfected cells, as reflected by low amplitude I_{ca}, exhibited less CDI than channel variants with higher mean current amplitudes (12).

In the present study, we also observed a similar relationship between current amplitude and CDI for channels containing the rat brain (rBA) α2.1 variant (Fig. 1). In transfected 293T cells, inactivation of currents carried by Ca²⁺ (I_{ca}) or Ba²⁺ (I_{ba}) was measured as I_{peak}/I_{peak}, which was the amplitude of the current at the end of a 2-s pulse normalized to the peak current amplitude. With minimal Ca²⁺ buffering of the intracellular recording solution (0.5 mM EGTA), we found that inactivation was significantly greater (smaller I_{peak}/I_{peak}) for large I_{ca} (>0.4 nA) than for small I_{ca} (<0.4 nA) (p < 0.01; Fig. 1, A and B) and that the increase in I_{ca} inactivation with current amplitude was significantly nonlinear (p < 0.05) (Fig. 1C). Because Ba²⁺ does not support calmodulin-dependent conformational changes that underlie CDI of voltage-gated Ca²⁺ channels (7, 33–36), Ca²⁺ currents carried by Ba²⁺ ions did not vary significantly with current amplitude (Fig. 1B). High concentrations (10 mM) of BAPTA in the intracellular recording solution, which block CDI of Ca²⁺ (7, 8), also prevented the current-dependent increase in inactivation (Fig. 1B), such that the relationship between current amplitude and inactivation for I_{ca}+ 10 mM BAPTA and for I_{ba} did not significantly deviate from a straight line (p = 0.83 for I_{ca}+10 BAPTA and p = 0.53 for I_{ba}, Fig. 1C). The positive slope for the current dependence of I_{ba} inactivation (0.02 ± 0.01) could have resulted from Ba²⁺-dependent effects on inactivation that have been described for L-type Ca²⁺ channels (37). It is not certain why the corresponding relationship for I_{ca} with 10 mM BAPTA exhibited a negative slope (−0.01 ± 0.01), although stimulatory effects of BAPTA on the amplitude of Ca²⁺ currents, have been reported in previous studies (38, 39). The difference between inactivation for I_{ca} and I_{ba}, which reflects the magnitude of CDI, was significantly greater for larger currents (~20%, p < 0.05, Fig. 1, A and B), primarily as a consequence of stronger, current-dependent inactivation of I_{ca}. Our analyses demonstrate that Ca²⁺ influx and its intracellular accumulation cause nonlinear increases in Ca²⁺ 1.1 inactivation and that current-dependent variations in CDI should be considered when evaluating factors that regulate this process.

To evaluate the modulatory potential of Ca²⁺ buffers on CDI, we compared the effects of BAPTA and EGTA at concentrations (0.5 mM) that are permissive for CDI. Whereas EGTA and BAPTA bind Ca²⁺ with nearly equal affinity, Ca²⁺ on- and off-rates for BAPTA are at least 100 times faster than for EGTA (40). Although BAPTA binds Ca²⁺ faster than EGTA, it will also retain it for shorter periods of time, such that CDI may be more evident with BAPTA than with EGTA. Consistent with this prediction, I_{peak}/I_{peak} was significantly smaller with BAPTA (0.5 mM) than with the same concentration of EGTA (~54% for I_{ca}+0.4 nA and ~60% for I_{ca}+0.4 nA, p < 0.01; Fig. 2A). For I_{ca}+0.4 nA, the rate of inactivation with BAPTA (0.5 mM) was significantly faster than with EGTA (~36%, Table 1). The effect of BAPTA was evident as an upward shift in the relationship between I_{ca} inactivation and current amplitude, which was significantly different from that with EGTA (p < 0.001, Fig. 2A). Greater inactivation with BAPTA than with EGTA was particularly apparent during repetitive depolarizations (Fig. 2B). With this voltage protocol and low EGTA (0.5 mM), I_{ca} underwent initial facilitation, which is also calmodulin-dependent (8), followed by inactivation. However, with BAPTA (0.5 mM), only strong inactivation of I_{ca} was observed, such that I_{ca} was reduced ~23% more by the end of the train compared with I_{ca} recorded with EGTA (Fig. 2B). These results show that the ability of I_{ca} to generate Ca²⁺ signals that cause CDI of
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Ca$_{2.1}$ is greater in the presence of fast Ca$_2^{+}$ buffers like BAPTA than with slow Ca$_2^{+}$ buffers like EGTA.

Effects of PV on CDI of Ca$_{2.1}$—To determine if Ca$_2^{+}$-buffering proteins might similarly regulate inactivation of Ca$_{2.1}$ Ca$_2^{+}$ currents, we investigated the effect of coexpressing Ca$_{2.1}$ with parvalbumin (PV, Fig. 3). We chose PV since its Ca$_2^{+}$-binding properties and effects on Ca$_2^{+}$ signals are well characterized (21). The EF-hands of PV can bind Ca$_2^{+}$ with high affinity and Mg$_2^{+}$ with lower affinity (31, 41). Under resting conditions, the concentration of Mg$_2^{+}$ in cells is generally far greater than that for Ca$_2^{+}$ (42), so that the rate of Ca$_2^{+}$ binding is slow because of a requirement for Mg$_2^{+}$ to first unbind (43). As a consequence, PV is considered a slow Ca$_2^{+}$ buffer in cells, with similar Ca$_2^{+}$ binding kinetics as EGTA (21).

Based on our results with EGTA and BAPTA, we expected that PV, like EGTA, should decrease $I_{Ca}$/ $I_{Ba}$ inactivation. Whereas this was true for $I_{Ca} < 0.4$ nA (~32% increase in $I_{res}$/ $I_{Ba}$ for PV-transfected cells, $p < 0.05$;
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**TABLE 1**

| \(I_{\text{Ca}}\) | Buffers | \(n\) | \(\tau\) (s) | \(p\) value (relative to Cav2.1 + 0.5 EGTA) |
|---|---|---|---|---|
| \(I_{\text{Ca}}<0.4\) nA | Cav2.1 + 0.5 EGTA | 12 | 1.237 ± 0.107 | - |
| | Cav2.1 + 0.5 BAPTA | 6 | 0.956 ± 0.088 | 0.11 |
| | Cav2.1 + 10 BAPTA | 18 | 1.533 ± 0.084 | <0.05 |
| | Cav2.1 + PV | 9 | 1.690 ± 0.177 | <0.05 |
| | Cav2.1 + CB | 17 | 1.884 ± 0.141 | <0.01 |
| | Cav2.1 + 0.5 EGTA | 7 | 1.123 ± 0.098 | - |
| | Cav2.1 + 0.5 BAPTA | 5 | 0.721 ± 0.064 | <0.05 |
| | Cav2.1 + 10 BAPTA | 13 | 1.771 ± 0.192 | <0.05 |
| | Cav2.1 + PV | 5 | 0.747 ± 0.104 | <0.05 |
| | Cav2.1 + CB\(^*\) | 11 | 1.109 ± 0.045 | 0.89 |

* \(I_{\text{Ca}}<0.4\) nA for cells cotransfected with Cav2.1 + CB represents \(I_{\text{Ca}}\) greater than 0.4 nA but less than 0.8 nA.

**FIGURE 3**

PV has opposite effects on inactivation of small and large amplitude \(I_{\text{Ca}}\). \(I_{\text{Ca}}/I_{\text{m}}\) was determined as in Fig. 1 for \(I_{\text{Ca}}\) of cells transfected with Cav2.1 alone (black, dashed) or cotransfected with PV (gray, solid) for \(I_{\text{Ca}}\) less than or greater than 0.4 nA. Representative normalized current traces are shown above. Intracellular solution contained 0.5 mM EGTA and extracellular solution contained 10 mM Ca\(^{2+}\). \(p\) values were determined from 18-20 cells per condition. \(I_{\text{Ca}}\) inactivation was determined as in Fig. 1 for \(I_{\text{Ca}}\) varied less with small \(I_{\text{Ca}}\) (Fig. 3B). The peak \(I_{\text{Ca}}\) and shape of the I-V curves for cells transfected with Cav2.1 alone or cotransfected with PV were not different for either small or large \(I_{\text{Ca}}\) (Fig. 3C), excluding the possibility that the dual effects of PV on \(I_{\text{Ca}}\) inactivation were caused by alterations in voltage-dependent activation of Cav2.1, or variability in channel expression between groups. Western blots confirmed that PV was highly expressed in cotransfected cells but not in cells transfected with Cav2.1 alone (Fig. 3D). The effects of PV on the current dependence of \(I_{\text{Ca}}\) inactivation were especially apparent during trains of repetitive stimuli (Fig. 4). In these experiments, inactivation of \(I_{\text{Ca}}\) in cells transfected with Cav2.1 alone varied less with peak \(I_{\text{Ca}}\) amplitude than during sustained test pulses (Fig. 1). Because Ca\(^{2+}\)-dependent facilitation as well as inactivation is evident with this voltage protocol, temporal overlap of both forms of Ca\(^{2+}\) regulation may have minimized the current dependence of \(I_{\text{Ca}}\) inactivation in cells transfected with Cav2.1 alone (Fig. 4D). However, in cells cotransfected with PV, the dependence of \(I_{\text{Ca}}\) inactivation on current amplitude was more pronounced (Fig. 4B). Consistent with results obtained with sustained test pulses (Fig. 3, A and B), PV inhibited inactivation of small \(I_{\text{Ca}}\) and had the opposite effect on large \(I_{\text{Ca}}\). The net effect of PV was to significantly augment differences in inactivation for \(I_{\text{Ca}}\) of different peak amplitudes (Fig. 4B).

The Ca\(^{2+}\)-buffering properties of PV could account for the suppression of inactivation of small currents, but we wondered if the opposite effect of PV on large currents could have resulted from Ca\(^{2+}\) unbinding from PV. Large \(I_{\text{Ca}}\) through Cav2.1 channels could rapidly saturate the EF-hand Ca\(^{2+}\) binding sites on PV, and subsequent Ca\(^{2+}\) release from PV might then facilitate CDI. At the concentration of free intracellular Mg\(^{2+}\) in our experiments (~1 mM), estimated rates of Ca\(^{2+}\) unbinding from PV (~0.9/s, Ref. 44) are consistent with the potential for Ca\(^{2+}\) release from PV to occur within the 2-s depolarizing pulse in our experiments (Fig. 3A). In this context, both the inhibitory and stimulatory effects of PV on \(I_{\text{Ca}}\) inactivation should depend critically on the ability of PV to bind Ca\(^{2+}\). To test this, we generated a PV construct with mutations in the second and third EF-hands (PV\(_{\text{CDEFF}}\)). Because these alter-
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FIGURE 4. PV alters CDI during repetitive stimuli. A and B, left panels, fractional current was determined as in Fig. 3A and plotted against time for cells transfected with Cav2.1 alone (A) or cotransfected with PV (B) for \(I_{Ca}\) of different amplitudes indicated in A. Right panels, fractional current for the last 10 pulses were averaged and compared for the different groups. Numbers of cells are shown in parentheses and \(p\) values from one-way analysis of variance are indicated.

FIGURE 5. Effects of PV require Ca\(^{2+}\)-binding to PV and are specific for CDI. A and B, left panels, \(I_{Ca}/I_{ph}\) was determined as in Fig. 1 for \(I_{Ca}\) less than or greater than 0.4 nA. Traces above show normalized \(I_{Ca}\) in cells transfected alone (black, dashed), cotransfected with PV (gray, solid, A), or cotransfected with PV (gray, solid, B). Right, relationship between % inactivation and \(I_{Ca}\) amplitude for cells transfected with Cav2.1 + PV (circles, A) or Cav2.1 + PV (squares, B). Dashed line represents data replotted from Fig. 1C. Smooth line shows fit from nonlinear (A) and linear (B) regression.
was not significantly different in cells transfected with Cav2.1 alone and those cotransfected with PV (p = 0.30) or CB (p = 0.54). The ineffectiveness of PV and CB in these experiments can be explained by a reliance of facilitation on local Ca\textsuperscript{2+} increases that are not able to be suppressed by even high concentrations of EGTA and BAPTA (8, 12, 48). These results also show that calmodulin was not likely to be a limiting factor contributing to the reduced CDI in cells expressing Cav2.1 with PV or CB. Taken together, our findings indicate that PV and CB modulate Ca\textsubscript{2.1} Ca\textsuperscript{2+} currents by regulating global Ca\textsuperscript{2+} microdomains that support CDI.

**DISCUSSION**

In the present study, the use of endogenous and exogenous Ca\textsuperscript{2+} buffers revealed new insights into the feedback regulation of Cav2.1 channels by Ca\textsuperscript{2+}. First, we confirmed and extended previous findings (12) that Cav2.1 inactivation increases nonlinearly with the amplitude of \(I_{Ca}\) an effect not observed when intracellular Ca\textsuperscript{2+} is buffered with BAPTA (10 mM) or when Ba\textsuperscript{2+} is the charge carrier. Second, BAPTA, at submaximal concentrations, strengthens the relationship between current amplitude and CDI compared with EGTA, perhaps because of its faster Ca\textsuperscript{2+} unbinding kinetics. Third, the Ca\textsuperscript{2+}-buffering proteins PV and CB also alter the Ca\textsuperscript{2+} current dependence of Cav2.1 inactivation. These findings illustrate how factors affecting Cav2.1 expression and Ca\textsuperscript{2+} homeostasis may dynamically regulate Cav2.1 properties and Ca\textsuperscript{2+} signaling in excitable cells.

**Differential Modulation of Cav2.1 Inactivation by Ca\textsuperscript{2+} Buffers**—Based on our results and those published previously, we propose a qualitative model to account for the Ca\textsuperscript{2+} dependence of Cav2.1 inactivation (Fig. 8). Unlike the relative invariance of the local Ca\textsuperscript{2+} microdomain associated with individual channel openings, Ca\textsuperscript{2+} pools that support CDI may be enhanced by larger \(I_{Ca}\) because of increased overlap of global Ca\textsuperscript{2+} signals emanating from neighboring channels (Fig. 8A). Small \(I_{Ca}\) may inactivate less than large \(I_{Ca}\) because, when not supported by multiple channels, these Ca\textsuperscript{2+} pools dissipate rapidly due to diffusion of Ca\textsuperscript{2+} (Figs. 1 and 8A). These Ca\textsuperscript{2+} gradients surrounding individual channels are collapsed by high concentrations of BAPTA (Fig. 1) or EGTA (8), thus abolishing the dependence of CDI on current amplitude (Figs. 1C and 8A). However, at concentrations of Ca\textsuperscript{2+} buffers that are permissive for CDI, Ca\textsuperscript{2+} unbinding from the buffer may paradoxically stabilize the Ca\textsuperscript{2+} pool supporting CDI (Fig. 8, B and C). Considering first the increased CDI seen with BAPTA compared with EGTA (0.5 mM, Fig. 2), the 100-fold faster association and dissociation rate of Ca\textsuperscript{2+} from BAPTA allows it to capture but also unload Ca\textsuperscript{2+} more rapidly than EGTA (40). Therefore, the time that Ca\textsuperscript{2+} will remain bound...
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Before dissociating is considerably less for BAPTA (~6–60 ms), than for EGTA (~700–2000 ms) (49), such that more rapid Ca\textsuperscript{2+} unbinding from BAPTA (0.5 mM) may cause greater CDI of both small and large \( I_{\text{Ca}} \) compared with the same concentration of EGTA (Figs. 2 and 88).

The dual effects of PV on \( I_{\text{Ca,2.1}} \) inactivation may result not only from its slow Ca\textsuperscript{2+} binding properties but also more limited mobility of PV relative to EGTA and BAPTA. Because of its larger size, PV has a diffusional range (~43 \( \mu \text{m} \text{s}^{-1} \)) that is about five times less than that for EGTA (~200 \( \mu \text{m} \text{s}^{-1} \)) (44). When \( I_{\text{Ca,2.1}} \) channels are sparsely distributed in the plasma membrane (small \( I_{\text{Ca}} \)), PV may have a net Ca\textsuperscript{2+} buffering effect in reducing CDI (Fig. 3), as PV may bind and shuttle Ca\textsuperscript{2+} away from channels prior to releasing it (17). However, with increased channel density (large \( I_{\text{Ca}} \), Ca\textsuperscript{2+}-saturated PV may unload Ca\textsuperscript{2+} within microdomains that support CDI of neighboring channels (Fig. 8C), therefore increasing inactivation of large amplitude \( I_{\text{Ca}} \) compared with in cells transfected with Ca\textsubscript{2.1} alone (Figs. 3 and 4). Although CB has fast Ca\textsuperscript{2+} binding kinetics more comparable to BAPTA than EGTA, CB also has considerably lower Ca\textsuperscript{2+} binding affinity (~1.5 \( \mu \text{M} \)) and diffusional range (~20 \( \mu \text{m} \text{s}^{-1} \)) than BAPTA (46, 50). Together, these factors may explain why CB behaved more like a slow Ca\textsuperscript{2+} buffer in inhibiting CDI only for low amplitude currents (Fig. 6).

For the following reasons, we consider the relatively modest effects of PV and CB on CDI in our experiments to underestimate the potential for these Ca\textsuperscript{2+} -buffering proteins to influence Ca\textsubscript{2.1} channels in neurons. First, the impact of PV was likely attenuated by the concentration of free Mg\textsuperscript{2+} in our intracellular recording solution (~1 mM). Because of the mixed Ca\textsuperscript{2+}/Mg\textsuperscript{2+} affinity of PV EF-hand (31, 51), PV would be occupied mainly by Mg\textsuperscript{2+} ions prior to evoking \( I_{\text{Ca}} \) in our experiments. Mg\textsuperscript{2+} unbinding from PV would therefore retard the rate of Ca\textsuperscript{2+} binding. Given that the estimated concentration of Mg\textsuperscript{2+} in neurons is ~0.3–0.6 mM (42, 52), we would predict more rapid Ca\textsuperscript{2+} binding by PV in neurons than in our transfected cell recordings, which should enhance its current-dependent modulation of CDI (Figs. 3 and 4). Second, PV and CB are found at concentrations in neurons (100 \( \mu \text{M} \)-5 mM) (53–56) that may exceed that in our transfected cells. Whereas Western blots of transfected cell lysates indicated strong levels of overexpressed PV and CB (Figs. 3D and 6A), heterogeneous levels of PV and CB between cells could have contributed to intercellular variability and weakened the average impact on CDI. Finally, washout of PV and CB from transfected cells into the recording pipette solution during whole cell recordings may have diminished the intracellular content of Ca\textsuperscript{2+}-buffering proteins. Whole cell patch clamp recordings of dentate granule cells in hippocampal slices suggest considerable dilution of endogenous CB within the first 5 min of obtaining whole cell configuration (57). Although we measured inactivation at similar time points between cells (~3–5 min after establishing whole cell mode) to limit variability, initial washout of PV or CB upon patch rupture could have significantly reduced the overall impact of these proteins on CDI.

**Ca\textsuperscript{2+} -buffering Proteins as Regulators of Ca\textsuperscript{2+} Signaling**—The effects of PV in altering feedback regulation of Ca\textsubscript{2.1} channels parallels its modulation of inositol 1,4,5-trisphosphate receptors (IP\textsubscript{R}s), which mediate Ca\textsuperscript{2+} release from intracellular stores (19, 20, 49). Overexpression of PV in *Xenopus* oocytes was found to stimulate Ca\textsuperscript{2+}-dependent activation of IP\textsubscript{R}s by facilitating Ca\textsuperscript{2+} diffusion between neighboring channels (19). That BAPTA, but not CB, replicated the effect of PV in this system was interpreted as a sign that the Ca\textsuperscript{2+} binding affinity and diffusional range of a Ca\textsuperscript{2+} -buffering protein were important determinants of the ability to enhance IP\textsubscript{R} activation by Ca\textsuperscript{2+} (19). The similar effects of PV in stimulating Ca\textsuperscript{2+} feedback of Ca\textsubscript{2.1} channels and IP\textsubscript{R}s may exemplify fundamental mechanisms controlling Ca\textsuperscript{2+} -dependent activation of other signaling pathways. In addition, the dual potential for PV to act as a passive Ca\textsuperscript{2+} buffer and a facilitator of intracellular and plasma membrane Ca\textsuperscript{2+} channels may contribute to the paradox that PV can be neuroprotective from pathological Ca\textsuperscript{2+} overloads in some neurons (58), but can also exacerbate excitotoxic cell death in others (59, 60).

Our findings also provide direct support for previous observations that Ca\textsuperscript{2+} -buffering proteins inhibit CDI of voltage-gated Ca\textsuperscript{2+} channels in neurons. Infusion of PV and CB in thalamic relay neurons caused an inhibition of CDI of high voltage activated Ca\textsuperscript{2+} current, although this effect was primarily on L-type channels (26). In addition, loss of CB from surviving granule cells isolated from the hippocampus of patients with mesial temporal lobe epilepsy correlated with increased CDI of voltage-gated Ca\textsuperscript{2+} currents, which was restored by inclusion of CB in the recording electrode (25). Interestingly, Chaudhuri et al. (24) found that Ca\textsubscript{2.1} channels in cerebellar Purkinje neurons show highly variable CDI, which may result partly from cellular variations in Ca\textsubscript{2.1} subunits expressed. Our results suggest that developmental or pathological alterations in PV and CB, as well as in the levels of Ca\textsubscript{2.1} expression, could add to the heterogeneity of Ca\textsubscript{2.1} inactivation in these and other neurons. Such variations in Ca\textsubscript{2.1} properties may be important for tailoring Ca\textsuperscript{2+} influx according to particular physiological contexts.

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