Ca²⁺ Regulation of Ca_v3.3 T-type Ca²⁺ Channel Is Mediated by Calmodulin

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ABSTRACT

Calcium-dependent inactivation of high voltage-activated Ca²⁺ channels plays a crucial role in limiting rises in intracellular calcium (Ca²⁺_i). A key mediator of these effects is calmodulin, which has been found to bind the C-terminus of the pore-forming α subunit. In contrast, little is known about how Ca²⁺_i can regulate low voltage-activated T-type Ca²⁺ channels. Using whole cell patch clamp, we examined the biophysical properties of Ca²⁺ current through the three T-type Ca²⁺ channel isoforms, Ca_v3.1, Ca_v3.2, or Ca_v3.3, comparing internal solutions containing 27 nM and 1 μ M free Ca²⁺. Both activation and inactivation kinetics of Ca_v3.3 current in 1 μ M Ca²⁺_i solution were more rapid than those in 27 nM Ca²⁺_i solution. In addition, both activation and steady-state inactivation

curves of Ca_v3.3 were negatively shifted in the higher Ca²⁺_i solution. In contrast, the biophysical properties of Ca_v3.1 and Ca_v3.2 isoforms were not significantly different between the two internal solutions. Overexpression of CaM₁₂₃₄ (a calmodulin mutant that doesn't bind Ca²⁺) occluded the effects of 1 μ M Ca²⁺_i on Ca_v3.3, implying that CaM is involved in the Ca²⁺_i regulation effects on Ca_v3.3. Yeast two-hybrid screening and co-immunoprecipitation experiments revealed a direct interaction of CaM with the carboxyl terminus of Ca_v3.3. Taken together, our results suggest that Ca_v3.3 T-type channel is potently regulated by Ca²⁺_i via interaction of Ca²⁺/CaM with the carboxyl terminus of Ca_v3.3.

Introduction

Voltage-activated Ca²⁺ channels (VACCs) are composed of pore-forming α_1 subunits and auxiliary subunits. Ten cDNAs encoding VACC α_1 isoforms were cloned and characterized in reconstitution systems. Based on their characteristics, Ca_v1.1–Ca_v1.4 are classified as dihydropyridine sensitive L-type Ca²⁺ channels, and Ca_v2.1–Ca_v2.3 are non-L-type Ca²⁺ channels. In comparison, Ca_v3.1–Ca_v3.3, open around the resting membrane potential of most cells, and hence, are classified as low voltage-activated (LVA) T-type Ca²⁺ channels (Perez-Reyes, 2003; Catterall, 2011; Zamponi et al., 2015).

When Ca²⁺ channels in *Paramecium* were biophysically characterized with different charge carriers, Ca²⁺ currents decayed more rapidly than Ba²⁺ currents. This effect on kinetics

is known as Ca²⁺-dependent inactivation (CDI). In addition, plots of the percent decay of peak currents against test potentials displays “U-shaped” voltage dependence, another hallmark of CDI. CDI has been regarded as a negative feedback limiting Ca²⁺ entry through Ca²⁺ permeable channels that prevents Ca²⁺ overloading, which could be toxic to cells (Eckert and Chad, 1984; Kass and Sanguinetti, 1984; Christel and Lee, 2012).

Electrophysiological studies have shown that CDI varies between different Ca²⁺ channel isoforms. L-type Ca_v1.2 and Ca_v1.3 showed strong CDI, regardless of Ca²⁺ buffering levels in the cytoplasmic side, while Ca_v2.1, Ca_v2.2, and Ca_v2.3 channels showed only weak or no CDI. CDI in the non-L type channels could be observed with 0.5 mM EGTA in the pipette solution, mimicking a physiologic condition with weak buffering of internal Ca²⁺, whereas it was not detected in high EGTA (or BAPTA) internal recording solutions that have high buffering capacity for internal Ca²⁺ (Lee et al., 2000; Liang et al., 2003; Christel and Lee, 2012).

The IQ motif in the C-terminus of Ca_v1.2 is known as one of the crucial structures determining CDI, displaying cooperation of its upstream pre-IQ and EF hand-like regions and its downstream CaM binding domain (de Leon et al., 1995; Peterson et al., 1999; Qin et al., 1999). The IQ-like motifs are well conserved among Ca_v1.2 and other HVA Ca²⁺ channels, and even certain

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ABBREVIATIONS: BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; CaM, calmodulin; CaM₁₂₃₄, calmodulin mutant lacking 4 Ca²⁺ binding sites; CDI, calcium dependent inactivation; C-terminus, carboxyl terminus; DMEM, Dulbecco's Modified Eagle Medium; EGTA, ethylene glycol tetraacetic acid; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HVA, high voltage-activated; LVA, low voltage-activated; TEACl, tetraethylammonium chloride; VACC, voltage-activated Ca²⁺ channel.

Na⁺ channels were found to interact with CaM, a calcium sensor ubiquitously expressed in most of eukaryotes. Point mutations of the motifs abolished CDI of HVA Ca²⁺ channels and changed the biophysical properties of certain Na⁺ channels (Peterson et al., 1999; Qin et al., 1999; Deschênes et al., 2002; Herzog et al., 2003; Ben-Johny et al., 2014). Alternatively, overexpression of CaM₁₂₃₄, a CaM mutant lacking the four Ca²⁺ binding sites abolishes CDI of Ca_v1.2 and Ca_v2.1 channels, supporting the hypothesis that CaM tethered to the IQ motif is crucially involved in channel CDI (DeMaria et al., 2001; Deschênes et al., 2002). Moreover, the CaM-induced CDI of Ca_v2.1 was specifically attributed to Ca²⁺ binding to the amino-terminal lobe of CaM, while its CaM-induced prepulse facilitation was attributed to Ca²⁺ binding to the carboxyl-terminal lobe of CaM (Lee et al., 1999, 2000; DeMaria et al., 2001; Liang et al., 2003). The lobe-specific roles of CaM in response to local or global Ca²⁺ changes have been found to distinctly contribute to CDI of VACCs (DeMaria et al., 2001; Liang et al., 2003; Dick et al., 2008; Ben-Johny et al., 2014). Even Na_v1.4 channels could show CDI in response to fast photo-releasing of caged Ca²⁺ in cytoplasm, broadening the notion that CDI might be a common mechanism of HVA Ca²⁺ channels and certain Na⁺ channel(s) for negative feedback regulation (Ben-Johny et al., 2014).

Previous studies attempted to detect CDI in native and cloned T-type channels which did not show clear CDI (Bossu et al., 1989; Klugbauer et al., 1999). For example, Ba²⁺ currents through T-type Ca_v3.1 and Ca_v3.2 were reported to decay more quickly than Ca²⁺ currents (Klugbauer et al., 1999; Serrano et al., 2000; Kaku et al., 2003). In addition, Ca²⁺/CaM in the patch pipette solution was reported to cause a negative shift in the activation curve of Ca_v3.2 channels, which was due to CaMKII mediated phosphorylation of specific sites in its II-III loop (Welsby et al., 2003). The carboxyl-terminus (C-terminus) of Ca_v3.2 was also reported to physically interact with calcineurin which was controlled by Ca²⁺/CaM (Huang et al., 2013).

In this study, we investigated whether T-type channel isoforms (Ca_v3.1–3.3) can be regulated by internal free [Ca²⁺] buffered to 27 nM and 1 μM. Among the three T-type channel isoforms, only Ca_v3.3 was regulated by an increase of internal free Ca²⁺. We present evidence indicating that the biophysical effects of Ca²⁺ on Ca_v3.3 is mediated by CaM.

Materials and Methods

Culture of HEK-293 Cells Stably Expressing Ca_v3.1, Ca_v3.2, and Ca_v3.3 Channel Isoforms. We previously reported the generation of HEK-293 cells stably transfected with cDNAs encoding rat Ca_v3.1 (GenBank accession number, AF027984), human Ca_v3.2 (AF051946) and rat Ca_v3.3 (AF086827) (Seo et al., 2013). The stable cell lines were cultured in DMEM supplemented with 10% FBS containing 1% penicillin-streptomycin, and 1 mg/ml G418 (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 95% air/5% CO₂. Several hours before patch clamp recordings, HEK-293 cells were dissociated with 0.05% trypsin-EDTA for ~3 minute (Invitrogen), and diluted with 10% FBS supplemented DMEM. They were triturated and plated on coverslips coated with 0.1 mg/ml poly-L-lysine (Sigma-Aldrich, St. Louis, MO) and maintained in a CO₂ incubator.

Electrophysiological Recording and Data Analysis. Ruptured patch clamp recordings were performed utilizing an Axopatch 200B patch-clamp amplifier (Molecular Devices, Foster City, CA), which was connected to a computer via a Digidata 1322A converter (Molecular Devices). The whole cell patch system and voltage-protocols

were controlled using pCLAMP 9.2 software (Molecular Devices). Recording pipettes were pulled from TW-150-3 capillary tubing (World Precision Instruments, Sarasota, FL) and fire polished. The resistance of the recording electrodes was 2~2.6 MΩ after being filled with internal solution. An external 10 mM Ca²⁺ (or Ba²⁺) solution used contained the following (in millimolars): 10 CaCl₂, (or 10 BaCl₂), 140 TEACl, 2.5 CsCl, 1 MgCl₂, 10 HEPES, 10 glucose, with pH adjusted to 7.3 with TEAOH. The capacitive transience was electronically compensated by 80%~85%. Values for series resistance and capacitance were obtained from direct readings of the amplifier. Based on the previous publications (Lu et al., 1994; Welsby et al., 2003), we prepared two pipette solutions with free Ca²⁺ concentrations buffered to 27 nM and 1 μM by mixing different combinations of CaCl₂ and BAPTA using Maxchelator (Table 1; Patton et al., 2004). In addition, the biophysical properties of the Ca_v3.3 channel were also compared using intracellular recording solutions containing 0.5 mM EGTA and 10 μM EGTA (Table 1; Lee et al., 2000; Liang et al., 2003).

As previously described (Seo et al., 2013), Ca_v3.3 currents were filtered at 1 kHz and sampled at 5 kHz, whereas Ca_v3.1 and Ca_v3.2 currents were filtered at 2 kHz and sampled at 10 kHz. Current amplitude and time constants were analyzed using Clampfit 9.2 software. Prism software (GraphPad, San Diego, CA) was used to fit activation and steady-state inactivation data with a Boltzmann equation. The smooth curve for channel activation was obtained by fitting the average data of chord conductance with a Boltzmann-Ohm equation:

$$G = 1/[1 + \exp\{(V_{50} - V)/S_{act}\}],$$

where V_{50} is the potential for half-maximal activation and S_{act} is the slope conductance. The curve for steady-state inactivation was derived by fitting normalized peak amplitudes against voltage with a Boltzmann equation:

$$I/I_{max} = 1/[1 + \exp(V_{50,inact} - V)/k],$$

where $V_{50,inact}$ is the potential for half-inactivation and k is the slope factor. Data are presented as mean ± S.D. Differences were evaluated for significance using Student's unpaired *t* tests or one-way ANOVA combined with Tukey's tests for multiple comparisons, with **P* < 0.05 and ***P* < 0.01.

Yeast Two-Hybrid Screening. We examined whether the cytoplasmic structures of human Ca_v3.2 and rat Ca_v3.3 interact with human calmodulin 2 (CaM2; NM001743.5) using the Matchmaker GAL4 yeast two-hybrid system according to the protocol supplied by the manufacturer (Clontech Laboratories, Mountain View, CA). The human CaM2 cDNA was inserted into prey vector pACT2, while the C-termini and cytoplasmic loops connecting domains I, II, III, and IV of human Ca_v3.2 and rat Ca_v3.3 were individually inserted into bait pGBKT7 vector. The prey plasmids and the bait plasmids were co-transformed into yeast strain AH109 and plated onto the SD medium lacking Leu, Trp, His, and Ade, but treated with X-Gal. The interaction between CaM2 and cytoplasmic structures of Ca_v3.2 and Ca_v3.3 could be determined by both the formation of colonies under high stringent conditions, and the blue color of the yeast colonies.

Co-immunoprecipitation between Rat Ca_v3.3 C-terminus and Human CaM2. Prior to co-immunoprecipitation (IP) experiments, the rat Ca_v3.3 C-terminus cDNA was fused with GST on the 5' end and tagged with FLAG on the 3' end using PCR methods. Similarly, the human CaM2 cDNA was fused with GST on the 5' end and tagged with MYC on the 3' end. The two cDNA constructs were subcloned into pGEX4T-1 plasmids for protein expression. The plasmids were transformed in *E. coli* Rosetta cells and positive colonies were selected and grown at 37°C in LB media supplied with ampicillin. Upon reaching an A₆₀₀ of 0.5~0.6, protein expression of FLAG-tagged Ca_v3.3 C-terminus and MYC-tagged CaM2 was induced by addition of 1 mM IPTG at 37°C for 5 hour. Cell lysis was performed by sonication, and cell debris was removed by centrifugation at 12,000g for 10 minute at 4°C. The

TABLE 1
Composition of internal solutions

Internal solution	Composition (in mM)	Reference
27 nM Ca ²⁺	115 CsCl, 1 MgCl ₂ , 0.9 CaCl ₂ , 20 HEPES, 11 BAPTA, 5 Mg-ATP (pH 7.3 with CsOH)	Lu et al. (1994)
1 μ M Ca ²⁺	115 CsCl, 1 MgCl ₂ , 8.8 CaCl ₂ , 20 HEPES, 11 BAPTA, 5 Mg-ATP (pH 7.3 with CsOH)	Welsby et al. (2003)
0.5 mM EGTA	135 CsMeSO ₃ , 5 CsCl, 0.5 EGTA, 10 HEPES, 1 MgCl ₂ , 4 Mg-ATP (pH 7.3 with CsOH)	Lee et al. (2000)
10 mM EGTA	135 CsMeSO ₃ , 5 CsCl, 10 EGTA, 10 HEPES, 1 MgCl ₂ , 4 Mg-ATP (pH 7.3 with CsOH)	Liang et al. (2003)

FLAG-tagged Ca_v3.3 C-terminus protein was incubated with Myc-tagged Cam2 protein overnight at 4°C in 1 ml immunoprecipitation buffer with 10 μ M CaCl₂ or 0 mM CaCl₂ plus 2 mM EGTA. The reaction solutions were then transferred to new tubes containing 100 μ l anti-Myc mouse mAb bounded protein G beads, followed by incubation overnight at 4°C.

The interaction of the full-length of rat Ca_v3.3 with CaM was also examined using HEK-293 cells and rat whole brain. All animal protocols were approved by the Guide for the Care and Use of Laboratory Animals of Sogang University (Seoul, Korea) and rats were housed in an SPF facility. All experiments were performed according to the guidelines of the Animal Care Committee of Sogang

University. HEK-293 cells were transiently transfected with either rat Ca_v3.3-pcDNA3 or pcDNA3 vector using jetPEI transfection reagent (Polyplus-transfection, Illkirch, France). Cells were lysed with CelLytic M lysis reagent (Sigma-Aldrich) containing protease inhibitor cocktail (Thermo, Rockford, IL). Rat brain lysate was prepared from 3-week old Sprague-Dawley rats and homogenized in 3 ml CelLytic MT lysis reagent containing protease inhibitor cocktail. Both lysates were incubated on ice for 30 minute and sonicated for 3 minute. Cell debris was removed by centrifugation at 12,000g for 5 minute at 4°C and supernatant was collected. The lysates of HEK-293 cells and rat brain tissues were mixed with 100 μ l of anti-Ca_v3.3 rabbit antibodies (Alomone Laboratories, Jerusalem, Israel) bounded protein

Fig. 1. Effects of Ca²⁺_i on the current-voltage relationships of Ca_v3.1, Ca_v3.2 and Ca_v3.3. (A–C) Representative current traces from HEK-293 cells stably expressing Ca_v3.1 (A), Ca_v3.2 (B), and Ca_v3.3 (C) were elicited by a voltage protocol consisting of serial step pulses from –70 to +50 mV by increments of 10 mV from a holding potential of –90 mV. Currents were measured in a 10 mM Ca²⁺_i recording solution with two internal solutions containing either 27 nM free Ca²⁺_i (left) or 1 μ M free Ca²⁺_i (right). (D–F) Current-voltage (*I*–*V*) relationships between two internal solutions containing 27 nM free Ca²⁺_i (open symbols) and 1 μ M free Ca²⁺_i (closed symbols). Current density (pA/pF) of Ca_v3.1 (D), Ca_v3.2 (E) and Ca_v3.3 (F) was obtained by dividing current amplitude by cell capacitance and their average values ($\pm$ S.D.) were plotted against test potentials ($n = 6–11$). (G–I) Activation and steady-state inactivation curves of T-type channel isoforms between the two internal solutions. Activation curves for Ca_v3.1 (G), Ca_v3.2 (H), and Ca_v3.3 (I) were from the chord conductance obtained by dividing current amplitude by driving force (reversal potential – test potentials), normalized to the peak conductance ($n = 6–12$). Channel availability at different potentials was obtained using an inactivation protocol which is composed of –20 mV step pulse following 10 second prepulses from –100 to –30 mV ($n = 6–8$). The current amplitudes measured at –20 mV test potential were normalized and plotted against test potential. The smooth curves were obtained by fitting the data with a modified Boltzmann equation. $V_{50,act}$ and $V_{50,inact}$ values and relevant slope factors (mean $\pm$ S.D.) for voltage-dependent channel activation and channel availability are listed in Table 2.

G beads, followed by overnight incubation at 4°C. Protein G beads were washed four times with IP binding buffer.

Bound proteins were recovered by boiling the beads in 1× SDS-PAGE sample buffer, separated on 10% polyacrylamide gels for SDS-PAGE, and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Immunoprecipitated proteins were detected by mouse monoclonal antibodies against FLAG and cMYC (Thermo), and rabbit polyclonal antibodies against Ca_v3.3 and CaM as primary antibodies. All other immunoblots were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and followed by the ECL method (Thermo).

Results

Regulation of Rat Ca_v3.3 T-type Channel by Internal Ca²⁺ (Ca²⁺_i). We recorded Ca²⁺ currents through T-type Ca_v3.1, Ca_v3.2 or Ca_v3.3 Ca²⁺ channels stably expressing in HEK-293 cells in 10 mM Ca²⁺ recording solution, using the whole cell patch clamp configuration. Free [Ca²⁺] in internal pipette solutions was adjusted to 27 nM and 1 μM after buffering with BAPTA (Table 1; Welsby et al., 2003). Serial Ca²⁺ current traces evoked from HEK-293 cells expressing each T-type channel isoform in response to a voltage-protocol were exhibited (Fig. 1, A–C). The average current-voltage (*I*-*V*) relationships of T-type Ca_v3.1, Ca_v3.2, and Ca_v3.3 using the two internal solutions were superimposed for comparison. The current density values of Ca_v3.3 evoked by only test pulses to –50 and –40 mV in 1 μM Ca²⁺_i solution appeared to be greater than those in 27 nM Ca²⁺_i solution (Fig. 1F), suggesting that the activation curve is negatively shifted by an increase of internal free Ca²⁺ from 27 nM to 1 μM (Fig. 1I). In comparison, the current density values of Ca_v3.1 and Ca_v3.2 at all the test potentials appeared to be similar between the two internal solutions (Fig. 1, D and E).

The activation curves and the steady-state inactivation curves for each T-type channel isoform were compared between the two internal solutions (Fig. 1, G and H). The activation and inactivation curves of Ca_v3.1 and Ca_v3.2 were similarly overlaid and showed no difference, suggesting that their voltage-dependent channel activation and steady-state inactivation properties do not differ between the two internal Ca²⁺ solutions (Table 2).

When the activation and inactivation curves of Ca_v3.3 were analyzed, the *V*_{50,act} values of activation curves are –31.5 ± 3.8 and –35.4 ± 4.1 (*n* = 10–12), and the *V*_{50,inact} values for steady-state inactivation curves are –58.3 ± 2.9 and –64.9 ± 3.1 (*n* = 6) between the internal solutions containing 27 nM and 1 μM free Ca²⁺, respectively. Statistical analysis showed

that both *V*_{50,act} and *V*_{50,inact} values with 1 μM Ca²⁺_i solution are different from corresponding values with 27 nM Ca²⁺_i solution, respectively (*P* < 0.05, 0.01; Table 2). These results indicate that both activation and inactivation curves for Ca_v3.3 are negatively shifted by an increase of internal free Ca²⁺ from 27 nM to 1 μM (Fig. 1I).

Acceleration of Ca_v3.3 Current Kinetics by an Increase of Free Ca²⁺_i. Among Ca_v3.1, Ca_v3.2, and Ca_v3.3 current traces (Fig. 1, A–C), only the Ca_v3.3 traces in 1 μM free Ca²⁺_i solution appear to be more transient than those in 27 nM free Ca²⁺_i solution. To visualize this point, we normalized and superimposed the Ca_v3.1–3.3 current traces elicited by a step pulse to 0 mV, at which each channel is fully activated (Fig. 2, A–C). Notably, Ca_v3.3 Ca²⁺ current in the higher Ca²⁺_i solution activated and inactivated faster than that in the lower Ca²⁺_i solution. The acceleration of activation and inactivation kinetics of Ca_v3.3 currents in the 1 μM Ca²⁺_i solution was observed at most of the test potentials examined (Fig. 2, Fa and b), illustrating the more transient kinetics in the 1 μM Ca²⁺_i solution (the right panel of Fig. 1C). Activation and inactivation kinetics of T-type channel currents are voltage-dependent at negative test potentials, but are voltage-independent at more positive potentials. Statistical analysis of the voltage-independent time constants for activation and inactivation are significantly different between the two internal Ca²⁺ solutions (Fig. 2F), supporting the finding that the both kinetics of Ca_v3.3 currents are accelerated in the higher Ca²⁺_i solution.

In contrast, the activation and inactivation current kinetics for Ca_v3.1 and Ca_v3.2 isoforms between the two Ca²⁺_i solutions are not statistically significantly different over various test potentials (Fig. 2, A, B, D, and E). Taken together, these findings suggest that current kinetics of Ca_v3.3 is uniquely accelerated by an increase of free Ca²⁺_i from 27 nM to 1 μM, but those of Ca_v3.1 and Ca_v3.2 are not affected.

Acceleration of Ca_v3.3 Current Kinetics in Other Conditions. The defining feature of CDI is that Ca²⁺ currents through VACCs decay more rapidly than Ba²⁺ currents (Eckert and Chad, 1984). Thus, we compared the current kinetics of Ca_v3.3 currents between different charge carriers of equimolar Ca²⁺ versus Ba²⁺, with a 0.5 mM EGTA internal solution (Table 1). The same voltage protocol for *I*-*V* was applied to elicit Ca_v3.3 currents in a 10 mM Ca²⁺ solution and then in a 10 mM Ba²⁺ solution (Fig. 3A). Comparison of the *I*-*V* curves showed that the magnitude of Ca_v3.3 Ba²⁺ currents were 40–43% greater than Ca_v3.3 Ca²⁺ currents at test potentials ranging between –20 and 0 mV. Compared with the *I*-*V* curve in 10 mM Ba²⁺ solution, the *I*-*V* curve in 10 mM Ca²⁺ was shifted

TABLE 2

Summary of biophysical properties of T-type channel isoforms with internal solution containing 27 nM or 1 μM free Ca²⁺.

	[Ca ²⁺] _i	Activation			Inactivation		
		<i>V</i> _{50, act} (mV)	Slope (<i>k</i>)	<i>n</i>	<i>V</i> _{50, inact} (mV)	Slope (<i>k</i>)	<i>n</i>
Ca _v 3.1 (α _{1G})	27 nM	–33.3 ± 3.4	5.9 ± 0.8	8	–62.3 ± 3.2	–4.0 ± 0.7	7
	1 μM	–34.2 ± 4.0	6.1 ± 0.8	9	–62.2 ± 0.9	–3.9 ± 0.7	6
Ca _v 3.2 (α _{1H})	27 nM	–36.4 ± 3.1	5.7 ± 0.9	6	–63.3 ± 2.9	–4.3 ± 0.7	6
	1 μM	–37.1 ± 3.4	5.6 ± 0.9	6	–63.5 ± 3.2	–4.1 ± 0.5	8
Ca _v 3.3 (α _{1I})	27 nM	–31.5 ± 2.4	5.3 ± 1.8	10	–59.3 ± 2.3	–4.4 ± 0.7	6
	1 μM	–35.5 ± 3.0	5.3 ± 0.9	12	–63.9 ± 2.7	–4.4 ± 0.7	6
+ CaM ₁₂₃₄	1 μM	–31.2 ± 3.9	6.1 ± 2.1	14	–60.3 ± 2.4	–4.7 ± 0.9	6

Data are presented as mean ± S.D. Significance levels of differences were evaluated using Student's *t* tests for Ca_v3.1 and Ca_v3.2 or one-way ANOVA, combined with Tukey's tests for Ca_v3.3: **P* < 0.05; ***P* < 0.01.

Fig. 2. Effects of Ca²⁺ on the current kinetics of Ca_v3.1, Ca_v3.2, and Ca_v3.3. (A–C) Representative Ca²⁺ current traces of Ca_v3.1 (A), Ca_v3.2 (B), and Ca_v3.3 (C) elicited by a step pulse to 0 mV from a holding potential of –90 mV were recorded with internal solutions containing 27 nM free Ca²⁺ (black) and 1 μM free Ca²⁺ (gray), respectively. The current traces in response to a step pulse to 0 mV among the superimposed traces in Fig. 1, A–C, were normalized in amplitude and then superimposed. (D–F) The activation and inactivation time constants of Ca_v3.1, Ca_v3.2, and Ca_v3.3 currents between internal solutions containing 27 nM free Ca²⁺ (white) and 1 μM Ca²⁺ (black). Activation (τ_{act}) and inactivation (τ_{inact}) time constants were obtained by fitting individual currents with an equation (built-in equation in pClamp using standard function and Chebyshev fitting methods) consisting of an exponential measuring activation and a second exponential measuring inactivation phases: $I(t) = A_1 \times \exp(-t/\tau_{inact}) + A_2 \times \exp(-t/\tau_{act}) + C$. Activation time constants (Da–Fa) and inactivation time constants (Db–Fb) of Ca_v3.1 (left, $n = 6–7$), Ca_v3.2 (middle, $n = 5–6$), and Ca_v3.3 (right, $n = 8–12$) currents elicited by an *I-V* protocol were plotted against test potentials. The limiting, voltage-independent time constants (τ) with errors were determined by fitting the average time constants with a Boltzmann equation and exhibited as bar graphs in the insets. The bars along the X-axis highlight the voltage independent range. The voltage-independent time constants between the low (○) and high (●) internal Ca²⁺ conditions were compared using Student's unpaired *t* tests. Significance levels are marked with asterisks as follows: **P* < 0.05; *****P* < 0.0001.

to positive potentials, possibly arising from surface charge screening effects of Ca²⁺ (Fig. 3B). Consistently, when the activation curves derived from chord conductance of the *I-V* data were analyzed, the $V_{50,act}$ values in 10 mM Ba²⁺ and Ca²⁺ solutions are -27.2 ± 5.4 and -32.0 ± 4.4 mV, respectively (*P* < 0.05, Student's unpaired *t* test, $n = 11$). Superposition of the Ca²⁺ and Ba²⁺ current traces elicited at 0 mV test potential illustrates how the Ca_v3.3 Ca²⁺ currents activated and inactivated faster than the Ca_v3.3 Ba²⁺ currents, supporting the suggestion that Ca_v3.3 has CDI (Fig. 3C). When activation and inactivation time constants of Ca_v3.3 currents in 10 mM Ba²⁺ or 10 mM Ca²⁺ solution were fitted with a Boltzmann equation, the voltage-independent time constant in 10 mM Ca²⁺ solution is statistically significantly smaller than those in 10 mM Ba²⁺ solution, supporting that the activation and inactivation kinetics of Ca_v3.3 current are faster in 10 mM

Ca²⁺ recording solution than 10 mM Ba²⁺ currents (insets of Fig. 3, D and E). These results imply that Ca²⁺ influx through Ca_v3.3 in 10 mM Ca²⁺ solution increases Ca²⁺_i, which subsequently causes both activation and inactivation kinetics of Ca_v3.3 currents to become more accelerated. These findings are consistent with the previous results that both activation and inactivation kinetics of Ca_v3.3 currents were accelerated by an increase of free Ca²⁺_i from 27 nM to 1 μM. Furthermore, the more rapidly decaying property of Ca_v3.3 currents in 10 mM Ca²⁺ than in 10 mM Ba²⁺ supports that Ca_v3.3 has CDI.

We next compared Ca_v3.3 Ca²⁺ currents using two internal solutions containing 0.5 and 10 mM EGTA (Table 1; Lee et al., 2000; Liang et al., 2003). The same voltage protocol for *I-V* was applied to evoke Ca_v3.3 Ca²⁺ currents in a 10 mM Ca²⁺ external solution (Fig. 3F). Comparing the results obtained between the two internal solutions revealed that the *I-V* relationship in

Fig. 3. Calcium-dependent inactivation of Ca_v3.3 channel by different conditions. The compositions of external and internal solutions are listed in Table 1. (A–E) Biophysical properties of Ca_v3.3 currents with 10 mM Ba²⁺_o or 10 mM Ca²⁺_o as charge carrier. (A) Representative current traces evoked by test pulses to –40, –20, 0, and +20 mV from a holding potential of –90 mV in 10 mM Ba²⁺ (left) and 10 mM Ca²⁺ (right). (B) *I*–*V* relationships of Ca_v3.3 between 10 mM Ba²⁺ (◇) and 10 mM Ca²⁺ (◆). (C) The Ba²⁺ and Ca²⁺ currents evoked by a test pulse of 0 mV among the raw traces (A) were normalized in amplitude and superimposed. (D–E) Time constants of Ba²⁺ and Ca²⁺ currents through Ca_v3.3. Time constants were determined by fitting current traces with a two exponential function (refer to the legend of Fig. 2). Between 10 mM Ba²⁺_o (○) or 10 mM Ca²⁺_o (●), the inactivation (D) and activation time constants (E) were plotted against test potentials ($n = 13$ –17) and fitted with a Boltzmann equation to obtain the voltage-independent tau values, which were exhibited as bar graphs in the insets. The bars along the X-axis highlight the voltage independent range. (F–J) Biophysical properties of Ca_v3.3 currents with 0.5 or 10 mM EGTA internal solution. (F) Representative current traces evoked by test pulses to –40, –20, 0, and +20 mV with 10 mM Ca²⁺ solution and internal solution containing 10 mM EGTA (left) or 0.5 mM EGTA (right). (G) *I*–*V* relationships of Ca_v3.3 Ca²⁺ currents with 0.5 mM (□) or 10 mM EGTA (■) internal solution. (H) The Ca²⁺ currents through Ca_v3.3 evoked by a test pulse of 0 mV among the raw traces shown in (F) were normalized to compare the biophysical effects of 0.5 mM (gray) and 10 mM (black) EGTA internal solutions. (I–J) Inactivation (I) and activation (J) time constants of Ca_v3.3 currents in two EGTA internal solutions. Currents evoked by an *I*–*V* protocol were fitted with double exponential function. The inactivation and activation time constants (mean $\pm$ S.D., $n = 9$ –15) in 0.5 (○) or 10 mM EGTA (●) internal solution were fitted with a Boltzmann equation to obtain the voltage-independent tau values, which were exhibited as bar graphs in the insets. The voltage-independent tau values were compared using Student's *t* tests. Significance levels of differences were evaluated using Student's unpaired *t* tests and exhibited as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

0.5 mM EGTA internal solution was negatively shifted, relative to that in 10 mM EGTA internal solution (Fig. 3G). Analysis of the *I*–*V* data showed that the $V_{50,act}$ values in the 0.5 and 10 mM

EGTA internal solutions are -29.9 ± 5.6 and -23.2 ± 6.4 mV ($P < 0.05$, Student's unpaired *t* test, $n = 12, 17$). When the Ca_v3.3 Ca²⁺ current traces elicited at 0 mV test potential were

superimposed, the Ca_v3.3 current trace in 0.5 mM EGTA internal solution activated and inactivated faster than that in a 10 mM EGTA internal solution (Fig. 3H). The effects on activation and inactivation kinetics of Ca_v3.3 currents in 0.5 mM EGTA internal solution were observed over a large range of test potentials (Fig. 3, I and J). This observation was supported by the analysis of the voltage-independent time constants are statistically different.

Fig. 4. Detection of CDI of Ca_v3.3 using a double-pulse protocol. (A) Whole cell Ca²⁺ currents were recorded from HEK-293 cells stably expressing Ca_v3.3 in response to a double-pulse protocol (B). (B) A double-pulse protocol consisting of serial pre-pulses ranging from -60 to +60 mV by a 20 mV (or 10 mV) increment, a brief gap, and a fixed test pulse to -20 mV. (C) Ca²⁺ currents evoked by the prepulses were normalized to the maximal current by the prepulse to -20 mV (○), while currents evoked by the fixed test pulse to -20 mV were normalized to the maximal current induced by the prepulse to -60 mV (●). The magnitude of normalized currents was plotted against prepulse potentials. The gray and dashed traces were evoked by preconditioning pulses to 0 and +60 mV, followed by a test pulse of -20 mV, respectively ($n = 5$).

Because Ca²⁺ influx through Ca_v3.3 would be less chelated in 0.5 mM EGTA solution than 10 mM EGTA solution, the cytoplasmic Ca²⁺ concentration would be increased more in the lower EGTA solution, resulting in accelerating of the activation and inactivation kinetics of Ca_v3.3 current. These findings are consistent with the results that the current kinetics of Ca_v3.3 was accelerated by an increase of free Ca²⁺_i from 27 nM to 1 μM.

Weak CDI of Ca_v3.3 Detected by a Double-Pulse Protocol. For certain HVA Ca²⁺ channels, another hallmark of CDI can be simply detected using a double-pulse voltage-protocol, because levels of inactivation induced by prepulses influence on the magnitude of currents at a subsequent test pulse (Budde et al., 2002; Christel and Lee, 2012). In this study, we thus examined whether the CDI of Ca_v3.3 can be detected using a double-pulse protocol. In 10 mM Ca²⁺_o external solution and 0.5 mM EGTA internal solution (Table 1), Ca_v3.3 Ca²⁺ currents were elicited by a double pulse protocol consisting of prepulses (from -60 to +60 mV) and a fixed test potential to -20 mV after a brief gap (Fig. 4B). The currents evoked by a fixed potential to -20 mV appeared to be biggest at a prepulse potential to -60 mV. The subsequent currents gradually reduced to become smallest (in gray) at a prepulse to 0 (or +10) mV (Fig. 4A). Interestingly, the next currents tended to be slightly increased in amplitude at stronger prepulses. The maximally increased current was induced by a prepulse to +60 mV which produced outward current (dashed) (Fig. 4A). The currents evoked by a fixed test potential was normalized to the biggest current at a prepulse potential to -60 mV. The percent amplitude of normalized currents at prepulses to 0 and +60 mV is $20.1\% \pm 0.87\%$ and $24.6\% \pm 0.35\%$ ($n = 5$; Fig. 4C). The current pre-conditioned by a prepulse to +60 mV is 4.5% greater than that by a prepulse to 0 mV, suggesting that Ca_v3.3 has relatively weak CDI. Consistently, the whole profile of normalized current amplitude plotted against pre-pulse potentials (Fig. 4C) is not like a typical bell-shape which is a hallmark CDI characteristic for certain HVA Ca²⁺ channels, because the inactivation rates of T-type Ca_v3.3 is relatively fast and strongly voltage-dependent (Frazier et al., 2001).

Involvement of Calmodulin in the Acceleration Effects on Ca_v3.3 Currents. We hypothesized that calmodulin (CaM) is involved in the acceleration effects on Ca_v3.3 current kinetics by an increment of free Ca²⁺_i, because CaM has been found to mediate CDI of HVA Ca²⁺ and certain Na⁺ channels. To test the hypothesis, we examined whether overexpression of the Ca²⁺-insensitive mutant (CaM₁₂₃₄) can abolish these effects. We thus transfected HEK-293 cells stably expressing Ca_v3.3 with CaM₁₂₃₄, and then measured Ca_v3.3 Ca²⁺ currents in 1 μM free Ca²⁺_i solution (right panel, Fig. 5A), of which representative current traces were exhibited together with the Ca²⁺ current traces in 27 nM free Ca²⁺_i solution (left panel) and 1 μM free Ca²⁺_i solution (middle panel).

Among the traces shown in Fig. 5A, the current traces in response to a step pulse to 0 mV were normalized and superimposed for comparison (Fig. 5B). The current kinetics of Ca_v3.3 in 1 μM free Ca²⁺_i solution were faster than those in 27 nM free Ca²⁺_i solution. Interestingly, overexpression of CaM₁₂₃₄ slowed down the accelerated current kinetics of Ca_v3.3 in 1 μM free Ca²⁺_i solution, becoming similar to the kinetics of Ca_v3.3 in 27 nM free Ca²⁺_i solution. The inactivation and activation time constants (τ_{inact} and τ_{act}) of Ca_v3.3 currents were analyzed and presented as bar graphs for comparison (Fig. 5, C and D), both of them of Ca_v3.3 current in 1 μM free Ca²⁺_i solution were different from those in

Fig. 5. Suppression of the Ca^{2+} effects on $\text{Ca}_v3.3$ by CaM_{1234} . (A) Representative $\text{Ca}_v3.3$ Ca^{2+} currents elicited in response to step pulses to -40 , -20 , 0 , and $+20$ mV from a holding potential of -90 mV in 10 mM Ca^{2+} solution. The Ca^{2+} current traces were recorded from HEK-293 cells stably expressing $\text{Ca}_v3.3$ with 27 nM Ca^{2+} internal solution (left), 1 μM Ca^{2+} internal solution (middle), and 1 μM Ca^{2+} internal solution plus CaM_{1234} (right). (B) Suppression of the high Ca^{2+} effects on $\text{Ca}_v3.3$ current kinetics by CaM_{1234} . The current traces in response to a step pulse to 0 mV among the current traces in (A) were normalized in amplitude and superimposed. Overexpression of CaM_{1234} strongly reversed the accelerated kinetics of $\text{Ca}_v3.3$ current in 1 μM Ca^{2+} solution, almost overlapping with the current trace recorded in 27 nM Ca^{2+} solution. (C–D) CaM_{1234} effects on the time constants of $\text{Ca}_v3.3$ currents. The currents shown in (B) were fitted with a two exponential function for activation and inactivation phases. The inactivation time constants (C) with 27 nM Ca^{2+} solution (white bar), 1 μM Ca^{2+} solution (gray bar), and CaM_{1234} plus 1 μM Ca^{2+} solution (black bar) are 61.6 ± 15.3 , 40.6 ± 9.9 , and 57.9 ± 9.8 , respectively ($n = 9$ – 12), and the activation time constants are 5.4 ± 1.7 , 3.6 ± 1.0 , and 5.0 ± 0.5 , respectively ($n = 9$ – 10). Significance of differences was evaluated with one-way ANOVA combined with Tukey's tests for multiple comparisons. Significance levels were marked as follows: $*P < 0.05$; $**P < 0.01$. (E) Effects of CaM_{1234} on the I - V relationships of $\text{Ca}_v3.3$. I - V relationships of Ca^{2+} currents recorded from HEK-293 cells expressing $\text{Ca}_v3.3$ alone with 27 nM ($\circ$) and 1 μM ($\bullet$) free Ca^{2+} solutions were compared with that of HEK-293 cells coexpressing $\text{Ca}_v3.3$ and CaM_{1234} ($\blacksquare$) with 1 μM Ca^{2+} internal solution. (F) CaM_{1234} effects on the activation and steady-state inactivation curves of $\text{Ca}_v3.3$. The activation and steady state inactivation curves were obtained by the same methods used in Fig. 1I. The $V_{50,\text{act}}$ values of activation curves with 27 nM Ca^{2+} solution ($\circ$), 1 μM Ca^{2+} solution ($\bullet$), and CaM_{1234} plus 1 μM Ca^{2+} solution ($\blacksquare$) are -31.5 ± 2.4 , -35.5 ± 3.0 , and -31.2 ± 3.9 , respectively ($n = 10$ – 14). The $V_{50,\text{inact}}$ values of inactivation curves are -59.3 ± 2.3 , -63.9 ± 2.7 , -60.3 ± 2.4 , respectively (mean $\pm$ S.D., $n = 6$). Significance of differences was evaluated with one-way ANOVA combined with Tukey's tests. The $V_{50,\text{act}}$ and $V_{50,\text{inact}}$ with 1 μM Ca^{2+} solution ($\bullet$) are different from those with 27 nM Ca^{2+} solution ($\circ$) or CaM_{1234} plus 1 μM Ca^{2+} solution ($\blacksquare$), respectively. The data are summarized in Table 2.

27 nM free Ca^{2+} solution or 1 μM free Ca^{2+} solution plus overexpression of CaM_{1234} , respectively (One-way ANOVA combined by Tukey's tests; $*P < 0.05$; $**P < 0.01$; ns, no significance; $n = 9$ – 10).

These results suggest that the accelerated kinetics of $\text{Ca}_v3.3$ current recorded in 1 μM Ca^{2+} solution was mediated by CaM , which can be strongly suppressed by overexpression of CaM_{1234} . The suppression effects by CaM_{1234} were observed at most test potentials. In addition, overexpression of CaM_{1234} also suppressed the negative shift of the activation and inactivation curves of $\text{Ca}_v3.3$ in 1 μM Ca^{2+} solution, becoming similar to those in 27 nM Ca^{2+} solution (Fig. 5F; Table 2). These findings support our hypothesis that the acceleration

in current kinetics and negative shifts in channel gating of $\text{Ca}_v3.3$ in 1 μM Ca^{2+} solution are mediated by CaM .

Direct Interaction of $\text{Ca}_v3.3$ with CaM . CDI in HVA Ca^{2+} channels and certain Na^{+} channels is known to be mediated by CaM binding to "IQ" or IQ-like motif in their C-terminal tails. However, there is no "IQ" or IQ-like motif in the cytoplasmic structures and C-terminus of rat $\text{Ca}_v3.3$. To look for interaction region(s) of rat $\text{Ca}_v3.3$ with CaM2 (human calmodulin 2), we employed Matchmaker Gal4 Two-Hybrid system for which CaM2 was used as a prey, while the cytoplasmic linkers connecting domains and the C-terminus of $\text{Ca}_v3.3$ were used as bait(s). Yeast two-hybrid screens showed that only the $\text{Ca}_v3.3$ C-terminus coexpressed with CaM2 induced yeast growth on high stringent

medium (lacking Leu, Trp, His, and Ade) and converted X-Gal to its blue metabolite. In contrast, the other cytoplasmic structures did not induce yeast growth under these conditions (Fig. 6A). These results indicate that Ca_v3.3 can interact with CaM2 in its C-terminus, implicating its action in the biophysical effects of internal free Ca²⁺ solutions on Ca_v3.3. In comparison, none of the cytoplasmic structures of human Ca_v3.2 were found to interact with CaM2, consistent with the lack of effects of altering internal free Ca²⁺ solutions on Ca_v3.2 gating.

We next employed co-immunoprecipitation (IP) assays to confirm that the C-terminus of rat Ca_v3.3 interacts with CaM2 and to examine whether the interaction is modulated by Ca²⁺. We overexpressed GST-Ca_v3.3-C-terminus-FLAG and GST-CaM2-MYC proteins, which were immunoprecipitated with the anti-MYC antibody in IP binding buffer solution with 10 μ M CaCl₂ or no added Ca²⁺ plus 2 mM EGTA. Labeling of the GST-Ca_v3.3-C-terminus-FLAG with anti-FLAG antisera was detected in both conditions (Fig. 6B), suggesting that the Ca_v3.3-C-terminus interacts with CaM2. In contrast, the immunoprecipitation from protein extracts containing GST-FLAG or GST-CaM2-Myc, which were used as negative controls, produced no cross-reactivity to the antisera (Fig. 6B). Although the

interaction appeared to be stronger in 10 μ M Ca²⁺ solution than Ca²⁺ free solution including 2 mM EGTA, the interaction between Ca_v3.3 C-terminus and CaM2 was detected even in Ca²⁺ free solution ($n = 3$).

To further determine whether rat Ca_v3.3 interacts with CaM, we performed co-immunoprecipitation from HEK-293 cells expressing the full-length cDNA of rat Ca_v3.3 or rat whole brain using rat Ca_v3.3 specific antibody. CaM was co-immunoprecipitated by anti-rat Ca_v3.3 antibodies from both HEK-293 cells expressing rat Ca_v3.3 and rat whole brain (Fig. 6C). In contrast, CaM signal was not detected by co-immunoprecipitation of lysates with empty pcDNA3 and protein G beads only. These results demonstrate that Cav3.3-CaM complexes can be detected both in vitro (HEK-293) and in vivo in the rat brain.

Discussion

We report that rat Ca_v3.3 T-type channel can be regulated by an increase of free intracellular Ca²⁺_i (27 nM – 1 μ M), showing an acceleration of activation and inactivation kinetics of Ca_v3.3 currents, and shifting the voltage-dependence of

Fig. 6. Interaction of the Ca_v3.3 with CaM. (A) In a highly stringent condition, yeast colonies were grown by a combination of the Ca_v3.3 C-terminus and CaM2, but not by any of the combinations of the other cytoplasmic structures and CaM2. Yeast colonies were not grown by any combinations of Ca_v3.2 cytoplasmic structures and CaM. (B) Interaction of the Ca_v3.3 C-terminus with CaM2 by co-immunoprecipitation. GST-Ca_v3.3 C-terminus-FLAG and GST-CaM2-MYC were incubated overnight, followed by immunoprecipitation with anti-MYC antibodies in IP buffer solution containing 10 μ M CaCl₂ (“+”) or 2 mM EGTA (“-”). The immunoprecipitated complexes were analyzed with Western blotting using anti-FLAG antibody (upper panel). Protein inputs for MYC-tagged CaM2 (middle panel), and FLAG-tagged GST and Ca_v3.3 C-terminus proteins (lower panel) were detected with anti-Myc-peroxidase and anti-FLAG-peroxidase, respectively. Co-immunoprecipitation experiments were repeated, showing similar results ($n = 3$). (C) Co-immunoprecipitation of full-length Ca_v3.3 and CaM in HEK-293 cells and rat brain. The lysates from HEK-293 cells overexpressing Ca_v3.3 and rat whole brain were co-immunoprecipitated with anti-Ca_v3.3 antibodies. Empty vector and protein G beads (mock) were used as negative controls for HEK-293 cells and rat brain tissues, respectively. The immunoprecipitated proteins were analyzed with Western blotting using anti-CaM antibody (lower panel). Expression of Ca_v3.3 in HEK-293 cells and rat brain was detected with anti-Ca_v3.3 antibodies (upper panel).

activation and steady-state inactivation toward negative potentials. These effects on $\text{Ca}_v3.3$ were abolished or strongly suppressed by overexpression of CaM_{1234} , suggesting that the mechanism underlying the internal Ca^{2+} regulation is mediated by CaM. Interaction of the $\text{Ca}_v3.3$ C-terminus with CaM was probed by yeast two-hybrid assays and co-IP experiments, supporting that CaM is the signaling molecule mediating in the internal Ca^{2+} regulation of $\text{Ca}_v3.3$.

On the contrary, we did not observe statistically significant differences in the biophysical properties of $\text{Ca}_v3.1$ and $\text{Ca}_v3.2$ between the two internal solutions. Dr. Barrett's group reported that the activation curve of $\text{Ca}_v3.2$ in an internal solution containing $1\ \mu\text{M}$ free Ca^{2+}_i and $2\ \mu\text{M}$ CaM was significantly shifted to the hyperpolarizing direction, compared with that in the internal solution containing $27\ \text{nM}$ free Ca^{2+}_i and $2\ \mu\text{M}$ CaM (Welsby et al., 2003). The underlying mechanism they found was that CaM activated by $1\ \mu\text{M}$ Ca^{2+}_i stimulated Ca^{2+} /CaM-dependent protein kinase II, leading to phosphorylation of certain residues in the II-III loop of $\text{Ca}_v3.2$. In the present study, no statistically significant shift in the activation curves of $\text{Ca}_v3.2$ was detected between internal solutions containing $27\ \text{nM}$ and $1\ \mu\text{M}$ free Ca^{2+} . We speculate that the different results might arise from internal pipette solution including or lacking CaM, which is required to stimulate Ca^{2+} /CaM-dependent protein kinase II. In regard to $\text{Ca}_v3.1$, the same group reported that $\text{Ca}_v3.1$ channel did not show statistically significant differences in its biophysical properties between the two internal solutions (Welsby et al., 2003). A similar result was obtained in the present study.

The finding that the inactivation kinetics of $\text{Ca}_v3.3$ currents were accelerated by the Ca^{2+}_i increase via activation of CaM interacting with the C-terminus of $\text{Ca}_v3.3$ is similar to the CDI mechanism for HVA Ca^{2+} channels, which is also mediated by CaM tethered to their C-termini. Although the Ca^{2+}_i increase shifted both the activation and steady-state inactivation curves of $\text{Ca}_v3.3$, the shift of the steady-state inactivation curve was greater than that of the activation curve (Fig. 1I; Table 2). These shifts reduced window current defined by the overlapping areas between the activation and inactivation curves, implying that Ca^{2+} influx through $\text{Ca}_v3.3$ would be decreased around resting membrane potential. This reduced window current would negatively regulate the functions of $\text{Ca}_v3.3$ which have been identified to crucially contribute to burst firing generation in the thalamic reticular nucleus, synchronized rhythmic activity in thalamic circuits, and spindle generation during sleep (Astori et al., 2011; Lee et al., 2014).

Electrophysiological characterization of the cloned three T-type channel isoforms ($\text{Ca}_v3.1$ – 3.3) revealed that the activation and inactivation kinetics of $\text{Ca}_v3.3$ current are about 5–10 fold slower than those of $\text{Ca}_v3.1$ and $\text{Ca}_v3.2$ (Perez-Reyes, 2003). Ca^{2+} influx through $\text{Ca}_v3.3$ with slowly inactivating current kinetics would contribute to the cytoplasmic Ca^{2+} increase more greatly than $\text{Ca}_v3.1$ and $\text{Ca}_v3.2$, which have faster inactivation kinetics. Therefore, $\text{Ca}_v3.3$ might be uniquely evolved for Ca^{2+} dependent negative feedback regulation in response to an increase in internal Ca^{2+} .

The hallmark properties of CDI for HVA Ca^{2+} channels includes more rapidly inactivating kinetics of Ca^{2+} currents than Ba^{2+} currents and the “U-shaped” voltage dependence of the decayed fractions of peak Ca^{2+} currents. When $\text{Ca}_v3.3$ channel currents were measured with $10\ \text{mM}$ Ca^{2+} or Ba^{2+} as the charge carrier, Ca^{2+} current elicited by a step pulse to $0\ \text{mV}$ was more

rapidly inactivated than Ba^{2+} current by the same pulse (Fig. 3). The more rapidly decaying property of $\text{Ca}_v3.3$ T-type channel in Ca^{2+} than Ba^{2+} is consistent with the hallmark CDI property of HVA Ca^{2+} channels. When the inactivation rates of $\text{Ca}_v3.3$ Ca^{2+} currents were plotted against test potentials, only a small “U-shaped” voltage dependence was observed (Fig. 4D). One of the possible reasons is that the inactivation rates of $\text{Ca}_v3.3$ are strongly voltage-dependent and coupled to activation, thereby obscuring the “U-shaped” Ca^{2+} entry dependence. Consistently, when the CDI of $\text{Ca}_v3.3$ was characterized using a double-pulse protocol, a plot of normalized currents evoked by a test potential against pre-pulse potentials weakly mimic a bell-shape (Fig. 4). The possible reason might be same for the small “U-shaped” voltage dependence of the inactivation rates of $\text{Ca}_v3.3$.

Interaction of CaM with the $\text{Ca}_v3.3$ C-terminus was probed by the two approaches of yeast two-hybrid assays and co-IP experiments. Our repeated yeast-two hybrid assays localized the interaction region of CaM to the 25 amino acid sequence (AHGLGPCPGPCPGPCPCPCPCPG) in the proximal region of rat $\text{Ca}_v3.3$ C-terminus. This sequence does not contain a consensus motif for CaM binding, and is not well conserved between species. Therefore it is possible that CaM is binding to other regions not detected in our experimental conditions. Notably, CaM2 could be immuno-precipitated by the $\text{Ca}_v3.3$ C-terminus not only in the presence of $10\ \mu\text{M}$ Ca^{2+} , but also in the absence of Ca^{2+} (plus $2\ \text{mM}$ EGTA) (Fig. 6B). Even though the immuno-precipitated signal in $10\ \mu\text{M}$ Ca^{2+} solution was weaker in Ca^{2+} free solution, the signal detected in Ca^{2+} free solution suggests that CaM is constitutively tethered to the $\text{Ca}_v3.3$ C-terminus. We suggest that the free Ca^{2+}_i increase from $27\ \text{nM}$ to $1\ \mu\text{M}$ causes free Ca^{2+} to bind CaM tethered to the $\text{Ca}_v3.3$ C-terminus, leading to conformational changes in CaM and then the C-terminus. The activated C-terminus

Fig. 7. A model to illustrate Ca^{2+}_i regulation of $\text{Ca}_v3.3$ channel. CaM is pre-associated with the C-terminus of $\text{Ca}_v3.3$ in a low Ca^{2+} condition (upper panel). An increase of Ca^{2+} in the cytoplasm allows Ca^{2+} to bind to CaM tethered to the C-terminus (lower panel). Ca^{2+} binding to CaM activates CaM of which conformational change tethered to the C-terminus induces the structural change of the C-terminus of $\text{Ca}_v3.3$ which is newly positioned to interact with the cytoplasmic region(s) of $\text{Ca}_v3.3$ domains involved in channel gating, resulting in acceleration of $\text{Ca}_v3.3$ current kinetics and negative shifts in activation and steady-state inactivation curves.

might then interact with other intracellular loops to induce conformation states of Ca_v3.3 with altered voltage gating. In this study, overexpression of CaM₁₂₃₄ was able to overcome the effects of wild-type CaM bound to the Ca_v3.3 C-terminus, and was also detected to interact with the C-terminus of Ca_v3.3 by yeast two hybrid screens (data not shown), supporting the idea that CaM is pre-associated with the C-terminus of Ca_v3.3. Therefore, we suggest that overexpression of CaM₁₂₃₄ mutant lacking Ca²⁺ binding sites could substitute for wild-type CaM tethered to the Ca_v3.3 C-terminus, and thereby occlude the effects of high internal Ca²⁺. Our interpretation based on the experimental results is illustrated in a simple model (Fig. 7).

Unexpectedly, overexpression of CaM₁₂₃₄ reduced the current density of Ca_v3.3 (Fig. 4B). According to previous studies, CaM has been suggested to act as an auxiliary subunit for many channels (Saimi and Kung, 2002). For example, expression of exogenous CaM was reported to upregulate the functional expression of Ca_v1.2 and $\alpha_2\delta$ via enhancing surface membrane targeting (Ravindran et al., 2008). Similarly, we speculate that endogenous CaM contributes to the membrane targeting of Ca_v3.3, which might be impaired by overexpression of exogenous CaM₁₂₃₄, resulting in a reduction in the current density of Ca_v3.3. Another possible explanation is that apo-CaM, but not CaM₁₂₃₄, modulates channel opening probability, as suggested in HVA Ca²⁺ calcium channels (Adams et al., 2014).

Taken together, our findings suggest that Ca_v3.3 is unique among T-type channel isoforms in displaying CDI properties including the accelerated inactivation kinetics and the negative shifts in channel gating properties in response to an internal Ca²⁺ rise. CaM, which seems to be pre-associated with the C-terminus of Ca_v3.3, plays a pivotal role as the signaling mediator to exert the CDI of Ca_v3.3.

Authorship Contributions

Participated in research design: K.-C. Kim, J.-H. Lee.

Conducted experiments: N. Lee, Jeong, K.-C. Kim, J.-A. Kim, Park, H.-W. Kang.

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