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Low-Mg\(^{2+}\) treatment increases sensitivity of voltage-gated Na\(^+\) channels to Ca\(^{2+}\)/calmodulin-mediated modulation in cultured hippocampal neurons

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MAGNESIUM (Mg\(^{2+}\)), a cofactor in numerous enzymes, plays a critical role in many physiological and pathological reactions. An abnormal Mg\(^{2+}\) level is associated with many diseases, including type 2 diabetes mellitus (19), Alzheimer’s disease (20), and epilepsy (4). Culture of hippocampal neurons in low-Mg\(^{2+}\) medium results in induction of continuous seizure activity, which is similar to induction of symptomatic epilepsy by status epilepticus in humans and animals, indicating that Mg\(^{2+}\) is essential for maintenance of electrical excitability in neurons. This in vitro model of epileptiform seizure activity is well suited to biochemical and electrophysiological investigations to elucidate the cellular mechanisms that underlie epileptogenesis and the spontaneous recurrent epileptiform discharge activity associated with epilepsy (4, 25). However, the underlying mechanism whereby low Mg\(^{2+}\) levels contribute to hyperexcitability of neurons has not been clarified.

Voltage-gated Na\(^+\) channels (VGSCs) are transmembrane proteins responsible for the action potential upstroke that initiates cell excitation and determines the velocity of impulse propagation. VGSC \(\alpha\)-subtypes Na\(_{V1.1}\), Na\(_{V1.2}\), Na\(_{V1.3}\), and Na\(_{V1.6}\) are abundantly expressed in the central nervous system (12, 28). The depolarization of neurons induces a transient, rapidly inactivating Na\(^+\) current (\(I_{\text{Na,t}}\)) followed by a persistent, noninactivating Na\(^+\) current (\(I_{\text{Na,p}}\)). Abnormal changes in \(I_{\text{Na,t}}\) and \(I_{\text{Na,p}}\) have been related to several mutations of VGSCs that cause epilepsy (8, 26, 27).

Calmodulin (CaM), an important regulator of ion channels, serves as a Ca\(^{2+}\) sensor for ion channels. CaM regulates several Na\(^+\) channel isoforms through binding to an IQ-like motif in the COOH terminus (2, 3, 17, 31). The CaM protein contains two lobes, each with two Ca\(^{2+}\)-binding sites. CaM mutations at Ca\(^{2+}\)-binding sites 1 and 2 in the NH\(_2\)-terminal (N) lobe (CaM\(_{12}\)) and mutations at Ca\(^{2+}\)-binding sites 3 and 4 in the COOH-terminal (C) lobe (CaM\(_{34}\)) have been used to investigate Ca\(^{2+}\)-dependent regulation of the CaV1.2 channel, and both N and C lobes have been shown to be of critical importance in CaM-dependent modulation of Ca\(^{2+}\) channels in our previous studies (13).

In a recent study we showed that expression of VGSC subtypes is upregulated in low-Mg\(^{2+}\) neurons and that this upregulation coincides with an increase in \(I_{\text{Na,p}}\) (14). In addition, we previously demonstrated that Ca\(^{2+}\)/CaM regulated Cav1.2 in a Ca\(^{2+}\)-dependent manner (13). Thus we speculate that Ca\(^{2+}\)/CaM may modulate VGSCs and that the modulation may be altered in low-Mg\(^{2+}\) neurons compared with normal neurons. In the present study we investigated the effects of Ca\(^{2+}\)/CaM on VGSCs and demonstrated that low-Mg\(^{2+}\) treat-
ment increased the sensitivity of VGSCs to the Ca$^{2+}$/CaM-mediated modulation. To our knowledge, this is the first study to demonstrate the concentration- and Ca$^{2+}$-dependent effects of wild-type CaM and CaM mutants with specific structural modifications on VGSCs in low-Mg$^{2+}$ neurons. Our data reveal novel features of the CaM-mediated modulation of VGSCs and provide a possible explanation for the seizure discharge observed in low-Mg$^{2+}$ neurons.

MATERIALS AND METHODS

Ethical statement. Considerable efforts were made to reduce animal suffering. All experiments were approved by the Animal Care Committee of China Medical University.

Preparation of glutathione S-transferase fusion proteins. Human CaM was expressed as a glutathione S-transferase fusion protein in Escherichia coli BL21 and purified using glutathione-Sepharose 4B (GE Healthcare). Point mutations in the Ca$^{2+}$-binding sites of CaM were introduced using the QuikChange site-directed mutagenesis kit (Qiagen). CaM$_{12}$ contained the mutations E31A and E67A, CaM$_{34}$ contained the mutations S101F and E140A, and CaM$_{234}$ contained the mutations E31A, E67A, S101F, and E140A (13).

Primary neuronal cell culture. Primary neuronal cells were cultured as previously described (14). Hippocampal neuronal cells from 1- to 3-day-old rats were dissociated in Hanks’ balanced salt solution containing 0.125% trypsin for 10 min at 37°C and then plated onto dishes in Dulbecco’s modified Eagle’s medium containing 15% fetal bovine serum and antibiotics. After 24 h, the medium was replaced with Neurobasal medium supplemented with 2% B27. Hippocampal neuronal cells were refreshed every 3–4 days. After 12 days in vitro, the hippocampal neurons were used for the following experiments.

Low-Mg$^{2+}$ treatment of cultured hippocampal neurons. The cultured hippocampal neurons were treated with low-Mg$^{2+}$ solution according to the method of Sombati and Delorenzo (25). For primary neuronal cell cultures, maintenance medium was replaced with physiological recording solution (145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 2 mM CaCl$_2$, 10 mM glucose, and 0.002 mM glycine, pH 7.3, with osmolarity adjusted to 290 ± 10 mosM using sucrose) with or without 1 mM MgCl$_2$ for 3 h. Continuous epileptiform high-frequency bursts were induced by exposure of neuronal cultures to physiological recording solution without added MgCl$_2$ (low-Mg$^{2+}$ condition) for 3 h; then the culture was exposed to physiological recording solution containing MgCl$_2$.

Electrophysiological recordings. The whole cell current-clamp method was used to record action potentials. The extracellular bath contained 135 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl$_2$, 0.33 mM Na$_2$HPO$_4$, 10 mM HEPES, and 5.5 mM glucose (pH 7.4). The pipette solution contained 50 mM K-aspartate, 20 mM KCl, 20 mM HEPES, 1 mM EGTA, 1 mM MgCl$_2$, 0.2 mM CaCl$_2$, 13.6 mM NaCl, and 3 mM K$_2$ATP$_3$ (pH 7.4). $I_{Na,p}$ was recorded in the cell-attached (1–3 min, basal activity) and inside-out (4–15 min) modes. At 4 min, the excised patch was moved to a small inlet of the perfusion chamber, which was connected to a microinjection unit. At 5–15 min, solutions containing wild-type CaM or CaM mutants were applied to the patch via the inlet. With use of this method, it is possible to strictly control drug concentrations. The extracellular bath contained 14 mM NaCl, 145 mM CsCl, 0.5 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5 mM HEPES, and 1 mM EGTA (pH 7.3). The pipette solution contained 150 mM NaCl, 5 mM CsCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES, and 2 mM CdCl$_2$ (pH 7.4). Ca$^{2+}$ concentration ($[$Ca$^{2+}$]) was calculated using a modification of the program of Fabiato and Fabiato (7). In our study the depolarization pulses to the neurons were from −100 to −60 mV at a holding potential of −100 mV for 200 ms at a rate of 0.5 Hz and digitized at 10 kHz. The mean current at 101–200 ms after the onset of the test pulses ($I$) was measured and divided by the unitary current amplitude ($I$) to yield $NP_o (N = N \times P_o \times i)$, where $N$ is the number of channels in the patch and $P_o$ is the time-averaged open-state probability of the channels. The basal channel activity was obtained by averaging $NP_o$ values for 3 min (90 pulses) in the cell-attached condition, and $NP_o$ values in test conditions were obtained as follows: the maximum mean $NP_o$ value from consecutive 3-min periods (90 pulses) with 1-min intervals in the inside-out mode was determined, then the maximum mean $NP_o$ values were normalized to the basal channel activity. All experiments were carried out at room temperature (22 ± 2°C), and data were analyzed using pClamp 10.0 software (Axon).

Western blot analysis. Neurons were exposed to Neurobasal medium supplemented with 2% B27 for 12 h after treatment with low-Mg$^{2+}$ [low-Mg$^{2+}$ condition (low-Mg$^{2+}$ neurons)] or with normal-Mg$^{2+}$ solution [control condition (normal neurons)]; then they were subjected to Western blot analysis as previously described (30). Equal amounts of proteins (40 µg) were subjected to SDS-polyacrylamide gel electrophoresis. Samples were then incubated overnight in Tris-buffered saline + Tween 20, 3% BSA, and 0.1% sodium azide containing the primary antibodies mouse anti-CaM (1:500 dilution; Santa Cruz Biotechnology) and rabbit anti-NaV1.1 (1:200 dilution), rabbit anti-NaV1.2 (1:200 dilution), rabbit anti-NaV1.3 (1:200 dilution), and rabbit anti-NaV1.6 (1:200 dilution; Millipore). There were no differences in GAPDH (as the reference protein) expression among the protein extracts. The ratio of VGSC or CaM to GAPDH was considered the relative protein expression of VGSC or CaM.

Immunofluorescence. Neurons were exposed to Neurobasal medium supplemented with 2% B27 for 12 h after treatment with low-Mg$^{2+}$ or normal-Mg$^{2+}$ solution; then they were studied using immunofluorescence as previously described (30). Briefly, the neurons were double-immunostained as follows: they were incubated first with primary antibodies [rabbit anti-NaV1.1 (1:100 dilution), anti-NaV1.2 (1:100 dilution), anti-NaV1.3 (1:100 dilution), and anti-NaV1.6 (1:100 dilution, Millipore) and mouse anti-CaM (1:100 dilution; Santa Cruz Biotechnology)] overnight at 4°C and then in fluorescein isothiocyanate-labeled goat anti-rabbit IgG and tetramethylrhodamine isothiocyanate-labeled goat anti-mouse IgG secondary antibodies (1:200 dilution; Santa Cruz Biotechnology) and rabbit anti-NaV1.2 (1:200 dilution); then they were studied using one-way ANOVA followed by Student’s t-test. Differences were considered significant when $P < 0.05$.

RESULTS

Low-Mg$^{2+}$ neurons exhibit epileptiform discharges. Using whole cell current-clamp analysis, we examined action potentials in cultured hippocampal neurons. The cultures prepared using our methods contained an enriched population of pyramidal glutamatergic neurons, with <10% inhibitory GABAergic cells, as determined in a previous study (1). Since the electrophysiological recordings were performed from the somatic region in pyramidal-like hippocampal neurons, most of our data were recorded from excitatory neurons. As shown in Fig. 1A, a representative normal neuron during 3 h of exposure to physiological recording solution containing 1 mM MgCl$_2$ revealed normal baseline activity with
Occasional action potentials. However, exposure of the cultured neurons to low-Mg²⁺ recording solution for 3 h resulted in continuous high-frequency epileptiform discharges. The resting potential of individual neurons was −70 to −65 mV. The spontaneous recurrent discharges in the low-Mg²⁺ neurons ranged from 8 to 20 Hz and lasted 10 s–3 min. Our data are in agreement with previous findings of epileptiform discharges in cultured hippocampal neurons exposed to low-Mg²⁺ solution (25).

In this study the current measured 101–200 ms after the onset of the test pulses was considered the \( I_{\text{Na,P}} \) (Fig. 1B). This current could be completely abolished by addition of 1 \( \mu \)M tetrodotoxin, a known voltage-gated Na⁺ channel blocker, indicating that \( I_{\text{Na,P}} \) is a tetrodotoxin-sensitive Na⁺ channel current (Fig. 1C). For the following experiments, all the representative traces of \( I_{\text{Na,P}} \) showed the last 100 ms (101–200 ms after onset of test pulses) of the original recording.

**Modulation of VGSC activity by CaM and its mutants requires ATP.** Our recent study showed that the \( N_P \) of \( I_{\text{Na,P}} \) was larger in low-Mg²⁺ than normal neurons. However, mean unit amplitude, slope conductance, and dwell time of \( I_{\text{Na,P}} \) did not differ between low-Mg²⁺ and normal neurons (13). Thus we mainly focused on \( N_P \) of \( I_{\text{Na,P}} \), which represented channel activity. As our previous studies showed that CaM and its mutants could modulate the activity of voltage-gated Ca²⁺ channels (13, 15), we investigated whether CaM and its mutants could modulate VGSC activity according to our previously described method for measuring \( N_P \) of the channel (14, 16).

We found that modulation of VGSCs by CaM and its mutants required ATP (Fig. 2), similar to the ATP dependence of CaM modulation in the CaV1.2 channel (13, 16, 29). In Fig. 2, B and D, \( N_P \) was minimal after patch excision. Application of CaM and its mutant CaM1234 alone resulted in a low level of channel activity [<20% (\( n = 5 \))] of basal activity at 1–3 min]. In contrast, Fig. 2A shows that application of wild-type CaM (1.4 \( \mu \)M) in the presence of 3 mM ATP at 80 nM Ca²⁺ after patch excision induced channel activity of 76.90 ± 3.88% (\( n = 5 \)) of basal activity. Similarly, application of CaM1234 (1.4 \( \mu \)M) in the presence of 3 mM ATP at 80 nM Ca²⁺ also resulted in recovery of channel activity to 74.42 ± 2.86% (\( n = 5 \)) of basal activity (Fig. 2C), whereas in the presence of ATP alone, channel activity was 11.60 ± 1.23% (\( n = 5 \)) of basal activity (Fig. 2E). Thus, channel activity was significantly higher after application of CaM and CaM1234 (1.4 \( \mu \)M) together with ATP (3 mM) than after application of CaM, CaM1234, or ATP alone (10.96 ± 1.30%) in normal neurons in the presence of 80 nM Ca²⁺ (Fig. 2F). Results were similar for application of CaM and CaM1234 in the presence of 500 nM Ca²⁺ and in the presence of 80 nM Ca²⁺ (see Supplemental Fig. S1 in Supplemental Material for this article, available at the Journal website). Our data show that modulation of VGSC activity by CaM and its mutants requires ATP.

**Modulating effects of CaM on VGSC activity in normal and low-Mg²⁺ neurons.** To determine the effects of CaM on VGSC channel activity, patches excised from normal and low-Mg²⁺ neurons were exposed to different concentrations of CaM. Figure 3, A–D, shows \( N_P \) values of \( I_{\text{Na,P}} \) recorded from patches excised from normal and low-Mg²⁺ neurons in response to 0.21 or 7 \( \mu \)M CaM in the presence of 3 mM ATP at 80 nM Ca²⁺. Figure 3E indicates that channel activity was

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**Figure 1.** Spontaneous action potentials in cultured hippocampal neurons exposed to low-Mg²⁺ solution. A: representative current-clamp recordings from a normal neuron showing occasional spontaneous action potentials (top trace) and induction of tonic high-frequency epileptiform bursts from a neuron after exposure to low-Mg²⁺ solution (bottom trace). Results are representative of ≥20 recordings. B: representative current traces from a normal neuron recorded in cell-attached mode. Depolarization pulses to the neurons were from −100 to −60 mV at a holding potential of −100 mV for 200 ms at a rate of 0.5 Hz. Current measured 101–200 ms after onset of test pulses was considered to be the persistent, noninactivating Na⁺ channel current \( I_{\text{Na,P}} \). C: blockade of the current by tetrodotoxin (TTX). This current in B could be abolished by 1 \( \mu \)M TTX.
CHANNELS

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Effects of CaM12, CaM34, and CaM1234 on VGSC activity in normal and low-Mg2+ neurons. To investigate the lobe-related effects of CaM regulation on VGSC activity, effects of the Ca2+-binding site-disabled mutants CaM12 (N-lobe mutant), CaM34 (C-lobe mutant), and CaM1234 (double mutant) were examined. NPo values of INa,p recorded from patches excised from normal and low-Mg2+ neurons in response to 0.7 μM CaM12, CaM34, or CaM1234 in the presence of 3 mM ATP at 80 nM Ca2+ are shown in Fig. 4, A–F. As indicated in Fig. 4, G–J, channel activity following application of different concentrations of CaM, CaM12, CaM34, and CaM1234 (0.007, 0.021, 0.7, 1.4, and 2.1 μM) was analyzed using the Hill equation. Channel activity in normal neurons exhibited a concentration-dependent increase after application of the three CaM mutants CaM12, CaM34, and CaM1234, as observed with CaM (n = 5). In addition, application of CaM12 and CaM34, as well as CaM

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Fig. 2. Modulation of voltage-gated Na+ channel activity by calmodulin (CaM) and the CaM mutant CaM1234 requires ATP. NPo values (where N is the number of channels in the patch and P, is the time-averaged open-state probability of the channels) for repetitive depolarization were calculated and plotted vs. time in cell-attached and inside-out (io) modes. A and B: 1.4 μM CaM + 3 mM ATP at 80 nM Ca2+ and 1.4 μM CaM alone at 80 nM Ca2+ were applied at times indicated above traces. C and D: 1.4 μM CaM1234 + 3 mM ATP at 80 nM Ca2+ and 1.4 μM CaM1234 alone at 80 nM Ca2+ were applied at times indicated above traces. E: 3 mM ATP alone at 80 nM Ca2+ was applied at times indicated above traces. F: analysis of channel activity in response to ATP in normal neurons. CaM and CaM12,34 (1.4 μM), together with ATP (3 mM), produced a significantly higher channel activity than CaM, CaM12,34, or ATP alone in normal neurons in the presence of 80 nM Ca2+. Values are means ± SE; n = 5 for each condition. **P < 0.01 vs. CaM or CaM12,34 alone; ##P < 0.01 vs. ATP.
Fig. 3. Concentration-dependent effects of CaM on voltage-gated Na\(^+\) channel activity in normal neurons and neurons exposed to low-Mg\(^{2+}\) solution (low-Mg\(^{2+}\) neurons). NP\(_o\) values for repetitive depolarization were calculated and plotted vs. time in cell-attached (1–3 min) and inside-out (4–15 min) modes. A range of CaM concentrations, together with 3 mM ATP, were applied at times indicated above traces. A and B: 0.21 µM CaM + 3 mM ATP at 80 nM Ca\(^{2+}\) was applied to patches excised from normal and low-Mg\(^{2+}\) neurons. Exemplar current traces are indicated for cell-attached mode (I) and with application of 0.21 µM CaM (II). C and D: 7 µM CaM + 3 mM ATP at 80 nM Ca\(^{2+}\) was applied to patches excised from normal and low-Mg\(^{2+}\) neurons. Exemplar current traces are indicated for cell-attached mode (I) and with application of 7 µM CaM (II). E: analysis of channel activity in response 0.007, 0.021, 0.07, 0.21, 0.7, 1.4, 2.1, 7, and 10 µM CaM in normal and low-Mg\(^{2+}\) neurons. Values are means ± SE; n = 5 for each condition. **P < 0.01; *P < 0.01 vs. normal neurons.
Fig. 4. Effects of the CaM mutants CaM12, CaM34, and CaM1234 on voltage-gated channel activity in normal and low-Mg\(^{2+}\) neurons. NPo values for repetitive depolarization were calculated and plotted vs. time in cell-attached (1–3 min) and inside-out (4–15 min) modes. Different concentrations of CaM12, CaM34, and CaM1234, together with 3 mM ATP, were applied at times indicated above traces. A and B: 0.7 \(\mu\)M CaM12 in the presence of 3 mM ATP at 80 nM Ca\(^{2+}\) was applied to patches excised from normal and low-Mg\(^{2+}\) neurons. C and D: 0.7 \(\mu\)M CaM34 in the presence of 3 mM ATP at 80 nM Ca\(^{2+}\) was applied to patches excised from normal and low-Mg\(^{2+}\) neurons. E and F: 0.7 \(\mu\)M CaM1234 in the presence of 3 mM ATP at 80 nM Ca\(^{2+}\) was applied to patches excised from normal and low-Mg\(^{2+}\) neurons. G–J: analysis of channel activity in response to wild-type CaM, CaM12, CaM34, and CaM1234 (0.007, 0.021, 0.7, 1.4, and 2.1 \(\mu\)M) in normal and low-Mg\(^{2+}\) neurons by the Hill equation with a Hill coefficient of 1. **P < 0.01; *P < 0.05 vs. normal neurons.
Fig. 5. Effects of Ca$^{2+}$/CALMODULIN on modulation of voltage-gated Na$^+$ channel activity in normal and low-Mg$^{2+}$ neurons. 

AP$_o$ values for repetitive depolarization were calculated and plotted vs. time in cell-attached (1–3 min) and inside-out (4–15 min) modes. 

A and B: 0.7 μM CaM with 3 mM ATP was applied at 80 nM Ca$^{2+}$/CALMODULIN as indicated above traces to normal and low-Mg$^{2+}$/CALMODULIN neurons. Exemplar current traces are indicated for cell-attached mode (I) and with application of CaM at 80 nM Ca$^{2+}$/CALMODULIN (II).

C and D: 0.7 μM CaM with 3 mM ATP was applied at 500 nM Ca$^{2+}$/CALMODULIN as indicated above traces to normal and low-Mg$^{2+}$/CALMODULIN neurons. Exemplar current traces are indicated for cell-attached mode (I) and with application of CaM at 500 nM Ca$^{2+}$/CALMODULIN (II).

E and F: analysis of channel activity in response to 80 and 500 nM Ca$^{2+}$/CALMODULIN in normal and low-Mg$^{2+}$/CALMODULIN neurons. Values are means ± SE; n = 5 for each condition. **P < 0.01; *P < 0.05 vs. control (80 nM Ca$^{2+}$/CALMODULIN).

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(0.021 and 0.7 μM), produced higher channel activity in low-Mg2⁺ than normal neurons (n = 5). Interestingly, CaM1234 (0.021, 0.7, and 1.4 μM) did not increase channel activity in low-Mg2⁺ neurons compared with normal neurons (n = 5), suggesting that increased channel activity after application of CaM in low-Mg2⁺ neurons requires binding of Ca²⁺ to at least one of the two lobes of CaM. Furthermore, Kᵦ values of the fitted curves with CaM, CaM12, and CaM34, but not CaM1234 (Ca²⁺-insensitive CaM), were much lower in low-Mg2⁺ than normal neurons (Fig. 4, G–J).

In general, the insensitivity of channel activity to CaM1234 regulation and the sensitivity of channel activity to CaM12 and CaM34, as well as CaM, in low-Mg2⁺ neurons indicate that functional Ca²⁺-binding sites in the N-lobe or C-lobe of CaM are required for modulation of VGSC activity in low-Mg2⁺ neurons.

**Effects of Ca²⁺ on CaM-mediated modulation of VGSCs in normal and low-Mg²⁺ neurons.** To further examine the modulating effects of Ca²⁺ on VGSC activity by wild-type CaM, different [Ca²⁺] were used during CaM application. NᵦP values of Nᵥ,P were recorded from patches excised from normal and low-Mg²⁺ neurons in response to 0.7 μM CaM in the presence of 5 mM ATP at 80 and 500 nM Ca²⁺ (Fig. 5, A–D). Channel activity in the normal neurons was significantly increased after application of 0.7 or 1.4 μM CaM at 500 nM Ca²⁺ compared with 80 nM Ca²⁺ (n = 5; Fig. 5E). However, in low-Mg²⁺ neurons, there was no obvious change in channel activity in response to CaM (0.7, 1.4, and 2.1 μM) at 500 or 80 nM Ca²⁺, and channel activity in 80 nM Ca²⁺ was already as high as that in 500 nM Ca²⁺ (n = 5; Fig. 5F). Furthermore, we measured intracellular [Ca²⁺] ([Ca²⁺]), in normal and low-Mg²⁺ neurons. Compared with normal neurons, the [Ca²⁺], relative fluorescence intensity of low-Mg²⁺ neurons was increased (n = 5; see Supplemental Fig. S2). Thus Ca²⁺ is involved in CaM modulation of VGSCs in normal and low-Mg²⁺ neurons. However, channel activity is more sensitive to Ca²⁺ regulation in low-Mg²⁺ than normal neurons.

**Expression of CaM and VGSC subtypes in normal and low-Mg²⁺ neurons.** To determine whether the increases in channel activity were related to changes in CaM expression, we performed Western blot analysis to investigate CaM expression in normal and low-Mg²⁺ neurons. As shown in Fig. 6A, expression of CaM protein was unchanged in neurons treated with low-Mg²⁺ solution (n = 6) compared with those treated with normal-Mg²⁺ solution. In a previous study we showed that expression of Naᵥ1.1, Naᵥ1.2, and Naᵥ1.3 protein was upregulated in low-Mg²⁺ neurons compared with normal neurons (14). In this study we examined differences in levels of expression among the VGSC subtypes. As shown in Fig. 6B, the level of expression was lowest for Naᵥ1.3 in normal and low-Mg²⁺ neurons (n = 8). Thus there were no significant differences in levels of expression for Naᵥ1.1, Naᵥ1.2, and Naᵥ1.6 between normal and low-Mg²⁺ neurons.

**Colocalization of CaM and VGSC subtypes in normal and low-Mg²⁺ neurons.** As the level of CaM expression in low-Mg²⁺ neurons was not different from that in normal neurons, we used double-label immunofluorescence experiments to investigate whether greater levels of CaM might be colocalized at the plasma membrane with VGSCs in low-Mg²⁺ neurons. We found that Naᵥ1.1, Naᵥ1.2, Naᵥ1.3, and Naᵥ1.6 colocalized with CaM in normal and low-Mg²⁺ neurons (Fig. 7). The colocalization was found mainly in the plasma membrane and proximal dendrites. We selected the yellow cells, in which both types of antigens were colocalized, and assessed their number per 100 cells. Although colocalization of Naᵥ1.6 and CaM was not significantly different between the two groups, the number of neurons that exhibited colocalization of CaM with Naᵥ1.1, Naᵥ1.2, or Naᵥ1.3 was greater in low-Mg²⁺ than normal neurons (n = 6; Fig. 8).
DISCUSSION

In this study, we focused on the effects of Ca^{2+}/CaM on VGSCs in low-Mg^{2+} neurons. We demonstrate the concentration- and Ca^{2+}-dependent effects of wild-type CaM and CaM mutants on VGSCs in low-Mg^{2+} neurons. Our main finding is that low-Mg^{2+} treatment increases the sensitivity of VGSCs to Ca^{2+}/CaM-mediated regulation in cultured hippocampal neurons.

ATP is required for the basal activity of VGSCs. We found that modulation of CaM and CaM mutants on the VGSC requires ATP. The role of ATP in regulation of Ca_{V}1.2 channel activity has been shown to be mediated by the protein kinase A-protein phosphorylation pathway (21, 22). Additionally, ATP may modulate channels independently of protein phosphorylation, via various mechanisms, including allosteric ef-

Fig. 7. Colocalization of CaM with VGSC subtypes in normal and low-Mg^{2+} neurons by immunofluorescence. A and B: protein distributions of Na_{V}1.1, Na_{V}1.2, Na_{V}1.3, and Na_{V}1.6 in normal and low-Mg^{2+} neurons. VGSCs were labeled with FITC (green), and CaM was labeled with tetramethylrhodamine isothiocyanate (red). Yellow staining indicates colocalization of VGSCs and CaM. Scale bars = 50 μm.
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Effects from ATP binding, changes in the actin cytoskeleton, and ATP-dependent phospholipases (18). We recently showed that ATP might regulate Cav1.2 channel activity by directly binding to the Cav1.2 channel in a dose-dependent manner (10). However, the exact mechanism underlying regulation of VGSCs by ATP is complicated and requires further investigation.

VGSC activity is more sensitive to regulation by CaM in low-Mg2+ than normal neurons. Our previous study showed that CaM could modulate the Cav1.2 channel in a concentration-dependent manner and with bell-shaped concentration-response relationships (13). Our finding in the present study of a concentration-response relationship of CaM in the modulation of VGSCs in normal and low-Mg2+ neurons (Fig. 3E) supports the hypothesis that modulation of different channels by CaM may take place via a number of mechanisms. One of our main findings is that application of CaM, including physiologically relevant concentrations, resulted in significantly higher levels of channel activity in low-Mg2+ neurons. Therefore, VGSC activity is more sensitive to regulation by CaM in low-Mg2+ than normal neurons, indicating a new feature of VGSC modulation in low-Mg2+ neurons.

VGSC activity is more sensitive to CaM-mediated Ca2+-dependent regulation in low-Mg2+ than normal neurons. Although many studies have revealed important aspects of the role of CaM in Ca2+-dependent facilitation and inactivation of ion channels, the specific role of each lobe of CaM in channel regulation remains controversial (5, 9, 24). Elevation of Ca2+ depolarized the voltage dependence of fast inactivation and slowed the fast inactivation kinetics of NaV1.1 (11). A previous study demonstrated dynamic changes in [Ca2+]i during prolonged electrographic seizure discharges in low-Mg2+ neurons, as the average [Ca2+]i levels increased from control levels of 150–200 nM to 450–600 nM (23). Thus Ca2+ could be a key element in modulation of VGSC activity by CaM.

It is noteworthy that channel activity in low-Mg2+ neurons was more sensitive to regulation by wild-type CaM and the CaM mutants CaM12 and CaM34. However, channel activity was insensitive to CaM1234 regulation, indicating that Ca2+ binding to CaM is required for the increased VGSC channel activity in low-Mg2+ neurons. Thus either the N-lobe or C-lobe of CaM is required for modulation of VGSCs in low-Mg2+ neurons. Interestingly, channel activity in response to CaM in normal neurons is significantly higher at 500 nm Ca2+, indicating a Ca2+-dependent effect of CaM modulation on VGSC activity in normal neurons (Fig. 5E). In addition, the channel activity produced by CaM in low-Mg2+ neurons at 80 nM Ca2+ was already as high as that produced at 500 nM Ca2+ (Fig. 5F). Therefore, VGSC activity is more sensitive to CaM-mediated Ca2+-dependent regulation in low-Mg2+ than normal neurons, suggesting that Ca2+ has been involved in modulation of VGSC activity.

More CaM can colocalize with VGSC subtypes at the plasma membrane in low-Mg2+ than normal neurons. CaM directly interacts with several Na+ channel isoforms via domains within their COOH terminus (2, 3, 17, 31). Patch-clamp experiments on HEK-293 cells stably transfected with human Nav1.1 showed that CaM overexpression increased peak current in a Ca2+-dependent manner (11). In addition, CaM overexpression accelerated the rate of fast inactivation, a finding that was reminiscent of the decrease in inactivation kinetics induced by IQ/EE or IQ/AA double mutations in the case of Nav1.6 or Nav1.4 (3, 17). Previously, we found upregulation of Nav1.1, Nav1.2, and Nav1.3 expression in low-Mg2+ neurons (14). In the present study we found that VGSC subtypes colocalized with CaM and that the number of neurons in which CaM colocalized with Nav1.1, Nav1.2, and Nav1.3 was higher in low-Mg2+ neurons, indicating that more CaM could colocalize with VGSC subtypes at the plasma membrane in low-Mg2+ than normal neurons.
A hypothetical model for Ca\(^{2+}\)/CaM modulation of VGSCs in low-Mg\(^{2+}\) neurons. Similar to the present study, in one of our recent studies we showed that the four VGSC subtypes colocalized with CaM in two rat models of epilepsy (30). In the present study, VGSC activity in normal and low-Mg\(^{2+}\) neurons was regulated by CaM in a concentration-dependent manner. In addition, channel activity was more sensitive to wild-type CaM regulation in low-Mg\(^{2+}\) than normal neurons. It is generally known that VGSC is an important target in epileptogenesis and that many antiepileptic drugs act via VGSC inhibition (6). Consequently, we propose a hypothetical model for CaM modulation of VGSCs (Fig. 9). In normal neurons, Ca\(^{2+}\)/CaM can bind to the corresponding binding sites of VGSCs at physiological [Ca\(^{2+}\)] (80–100 nM), resulting in a normal level of channel activity; however, in low-Mg\(^{2+}\) neurons, [Ca\(^{2+}\)]\(_i\) is increased compared with normal neurons, and more CaM can colocalize with VGSCs on the neuronal membrane; therefore, more Ca\(^{2+}\)/CaM may bind to corresponding binding sites of VGSCs, leading to elevated channel activity. Meanwhile, VGSC activity in low-Mg\(^{2+}\) neurons is more sensitive to Ca\(^{2+}\)/CaM regulation. Thus all these factors may result in hyperexcitability in low-Mg\(^{2+}\) neurons.

**Conclusions.** The most intriguing finding of the present study is that VGSC activity is more sensitive to Ca\(^{2+}\)/CaM regulation in low-Mg\(^{2+}\) than normal neurons, providing a possible explanation for the seizure discharge in low-Mg\(^{2+}\) neurons. As a core regulating factor, CaM connects the functional roles of the three main intracellular ions, Na\(^{+}\), Ca\(^{2+}\), and Mg\(^{2+}\), by modulating VGSCs. Thus low-Mg\(^{2+}\) neurons may be useful for screening antiepileptic drugs that act on CaM and specific VGSC subtypes. Collectively, this study reveals novel features of CaM-mediated regulation of VGSCs in low-Mg\(^{2+}\) neurons and provides insights into the modulation of VGSCs underlying hippocampal excitation.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


