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Prolonged activation of the *N*-methyl-D-aspartate receptor–Ca^{2+} transduction pathway causes spontaneous recurrent epileptiform discharges in hippocampal neurons in culture

(epilepsy/calcium/epileptogenesis/neuronal plasticity)

ROBERT J. DELORENZO*†‡§, SHUBHRO PAL*, AND SOMPONG SOMBATI*

Departments of *Neurology, †Pharmacology and Toxicology, and ‡Biochemistry and Molecular Biophysics, Medical College of Virginia of Virginia Commonwealth University, Richmond, VA 23298

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ABSTRACT **The molecular basis for developing symptomatic epilepsy (epileptogenesis) remains ill defined. We show here in a well characterized hippocampal culture model of epilepsy that the induction of epileptogenesis is Ca^{2+}-dependent. The concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was monitored during the induction of epileptogenesis by prolonged electrographic seizure activity induced through low-Mg^{2+} treatment by confocal laser-scanning fluorescent microscopy to directly correlate changes in $[Ca^{2+}]_i$ with alterations in membrane excitability measured by intracellular recording using whole-cell current–clamp techniques. The induction of long-lasting spontaneous recurrent epileptiform discharges, but not the Mg^{2+}-induced spike discharges, was prevented in low-Ca^{2+} solutions and was dependent on activation of the *N*-methyl-D-aspartate (NMDA) receptor. The results provide direct evidence that prolonged activation of the NMDA–Ca^{2+} transduction pathway causes a long-lasting plasticity change in hippocampal neurons causing increased excitability leading to the occurrence of spontaneous, recurrent epileptiform discharges.**

Epilepsy or the occurrence of spontaneous recurrent epileptiform discharges (SREDs, seizures) is one of the most common neurological conditions, affecting more than 2% of children and 1% of adults (1). Between 30% and 50% of epilepsy is symptomatic (1, 2), being caused by a known etiology that produces a permanent plasticity change in a previously normal brain, causing recurrent seizures (3). Much of our current knowledge concerning the pathophysiology of epilepsy has been derived from studies of human brain tissue from patients undergoing epilepsy surgery and various animal models of epilepsy (4–7). In several models of epilepsy, SREDs can be induced to occur for the life of the animal or preparation. The process of inducing SREDs or symptomatic epilepsy in previously normal neuronal networks is called epileptogenesis.

Calcium is a major second-messenger system that regulates many neuronal processes (8), and alterations in calcium homeostasis have been implicated in the induction of epileptogenesis (9). Furthermore, indirect evidence has suggested that *N*-methyl-D-aspartate (NMDA) receptor activation may contribute to the induction of altered neuronal excitability in the kindling (10), hippocampal slice (11), and hippocampal neuronal culture (7) models of epilepsy. Thus, it is important to determine whether the induction of epilepsy is clearly dependent on elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and NMDA receptor activation by directly measuring both $[Ca^{2+}]_i$ and neuronal excitability during epileptogenesis.

This investigation was initiated to determine whether the induction of epileptogenesis in a well characterized *in vitro* hippocampal neuronal culture (HNC) model of epilepsy (7) was calcium-dependent. This model of epilepsy is well suited to study the role of calcium in epileptogenesis, since it utilizes an episode of continuous seizure activity for 3 hr to induce permanent plasticity changes in the neurons that result in SREDs for the life of the preparation. Status epilepticus (SE; continuous seizures activity for 30 min or longer) has been shown to cause epilepsy in humans (12) and animals (13, 14). Thus, the HNC model of symptomatic "epilepsy" induced by 3 hr of prolonged electrographic seizure activity is similar to the induction of symptomatic epilepsy by status epilepticus in man and animals. This model also offers the ability to directly quantitate $[Ca^{2+}]_i$ while simultaneously pharmacologically regulating calcium channels and performing electrophysiological studies to monitor the development of SREDs.

This study demonstrates that prolonged elevations in $[Ca^{2+}]_i$ induce persistent plasticity changes in hippocampal neurons in culture that eventually induce epileptogenesis. We show that elevations in $[Ca^{2+}]_i$ mediated by NMDA receptor activation play a role in inducing SREDs. Conversely, inhibition of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, or metabotropic receptors or L-type voltage-gated Ca^{2+} channels did not block the generation of these epileptiform discharges. The elevation of $[Ca^{2+}]_i$ produced during SE in the presence of NMDA receptor inhibition was not sufficient to induce epileptogenesis, providing evidence for a role of the NMDA receptor–Ca^{2+} transduction pathway in causing spontaneous recurrent epileptiform discharges.

MATERIALS AND METHODS

Hippocampal Cell Culture. Primary hippocampal cultures were prepared from hippocampal tissue isolated from 2-day postnatal Sprague–Dawley rats and maintained in culture for up to 3 weeks according to previously documented procedures routinely performed in our laboratory (15, 16). The hippocampal cells were plated at a density of 2×10^5 cells per cm^2 onto a confluent glial support layer or on Matrigel-coated (Collaborative Research) dishes.

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; APV, 2-amino-5-phosphonovaleric acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline; NMDA, *N*-methyl-D-aspartate; SRED, spontaneous recurrent epileptiform discharge; SE, status epilepticus; TTX, tetrodotoxin.

§To whom reprint requests should be addressed at: Department of Neurology, Virginia Commonwealth University, P.O. Box 980599, Richmond, VA 23298. e-mail: rdeloren@hsc.vcu.edu.

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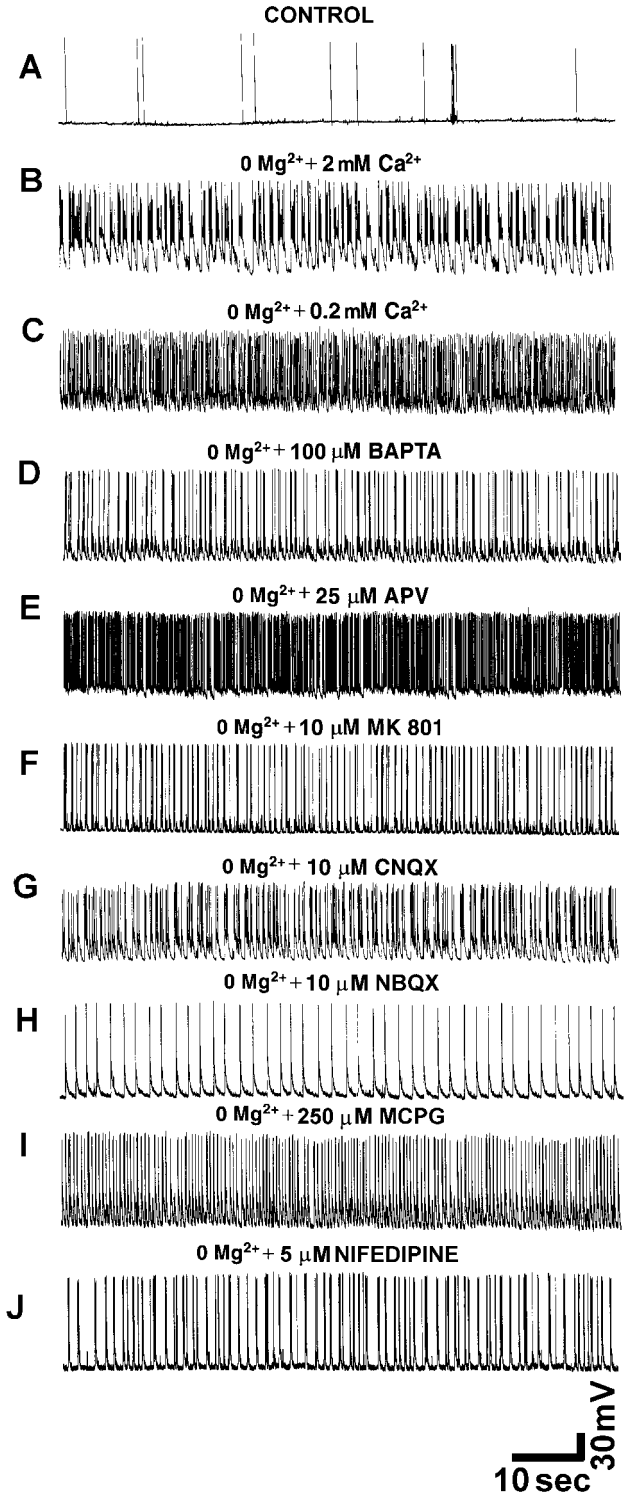

FIG. 1. Induction of continuous seizure activity (status epilepticus) in cultured hippocampal neurons for 3 hr in the presence and absence of several neuropharmacological agents. Intracellular recordings were obtained from hippocampal pyramidal neurons before, during, and after a 3-hr low-Mg^{2+} treatment employing established procedures (7). The recordings shown were representative of more than 10 independent experiments for each condition. (*A*) A representative intracellular recording from a control neuron showing occasional spontaneous action potentials. The resting potential of individual neurons ranged from −65 to −50 mV. Recording from more than 100 control neurons, no spontaneous seizure activity was observed. (*B–J*) Representative intracellular recordings during low-Mg^{2+} treatment in normal (2 mM) (*B*) or low (0.2 mM) Ca^{2+} (*C*), or various neuropharmacological agents (see Fig. 2 *D–J*). During the low-Mg^{2+} treatment the cells developed longer-duration synaptic potentials and multiple action potentials, evolving into continuous tonic high-frequency (4–20 Hz) burst discharges. The frequency of burst discharges varied from cell to cell. However, all the conditions shown in *C–J* manifested 3 hr of continuous seizure discharge. The low Ca^{2+} 0.2 mM (*C*), APV 25 μM (*E*), and MK801 10 μM (*F*) treatments consistently produced higher-frequency discharges. NBQX 10 μM (*H*) and 5 μM nifedipine (*I*) produced slightly lower-frequency discharges. After readdition of normal Mg^{2+} levels to the medium, all spontaneous continuous discharges attenuated. SE produced less than 10% neuronal cell death under these conditions (7).

Electrophysiological Recordings. Electrophysiological recordings were performed as discussed previously (15, 16). Briefly, electrophysiological studies were initiated by replacing the culture medium with a defined recording solution and in the presence or absence of Mg^{2+} as described previously (7). Cultures were transferred to the stage of an inverted microscope (Nikon Diaphot) and subjected to various experimental protocols while being monitored continuously by patch–clamp electrodes utilizing the whole-cell current–clamp recording mode. The chamber was perfused continuously with the recording medium and aspirated at the rate of 0.5 ml/min. Drugs were dissolved in the recording medium and applied by perfusion to study their effects on seizure discharges. All experiments were performed at 37°C.

Calcium Imaging. $[Ca^{2+}]_i$ measurements were performed according to Limbrick *et al.* (17) with minor modifications. The hippocampal cells were loaded with 1 μM indo-1 AM in the recording solution for 1 hr at 37°C. Cells were then rinsed with the recording solution and incubated for an additional 15 min to allow cleavage of the indo-1 AM ester to the free acid by the cellular esterases. $[Ca^{2+}]_i$ was measured by using the ratio program of either the image- or point-scan analysis mode of a confocal ACAS Ultima Interactive Laser Cytometer (Meridian Instruments, Lansing, MI). To excite indo-1, a 100-mW, 360-nm line of argon laser was passed through an Olympus D Plan Apo UV 40× short-working-distance objective mounted on an Olympus IMT-2 inverted microscope (New Hyde Park, NY). Emission wavelengths were passed through a 225-μm pinhole aperture before reaching the photomultiplier tubes (PMTs). Photocurrents from each PMT were then measured by a computer-driven data acquisition system. The ratio of emitted intensity at 405 nm (indo-1-Ca^{2+} complex) and 485 nm (free indo-1) was monitored to indicate relative free $[Ca^{2+}]_i$. Pyramidal cells were chosen for analysis based on their morphology. Absolute free $[Ca^{2+}]_i$ was estimated by comparison of fluorescence ratio values to the values obtained by generating a Ca^{2+} calibration curve as described previously (17). All experiments were performed in the defined recording solution at 37°C employing constant-temperature-heated stage.

RESULTS

Hippocampal neuronal cultures were prepared from hippocampal tissue isolated from 2-day postnatal Sprague–Dawley rats, as described previously (15, 16). Hippocampal cells were plated at a density of 2×10^5 cells per cm^2 onto a confluent glial support layer. Exposure to medium without Mg^{2+} for 3 hr caused 3 hr of continuous epileptiform activity (SE) (Fig. 1*B*). Exposure of neurons to 2 hr or less of low Mg^{2+} medium did not produce persistent SREDs (data not shown), indicating that prolonged epileptiform activity was required to induce SREDs. After exposure to low Mg^{2+}, the cultures were returned to normal Mg^{2+} and developed persistent plasticity changes and manifested SREDs for the life of the neurons in culture (2–3 weeks) (7). The SREDs that developed in this model of epilepsy were shown previously to represent synchronous epileptiform discharges on large populations of neurons (7). Intracellular recordings using whole-cell current–clamp

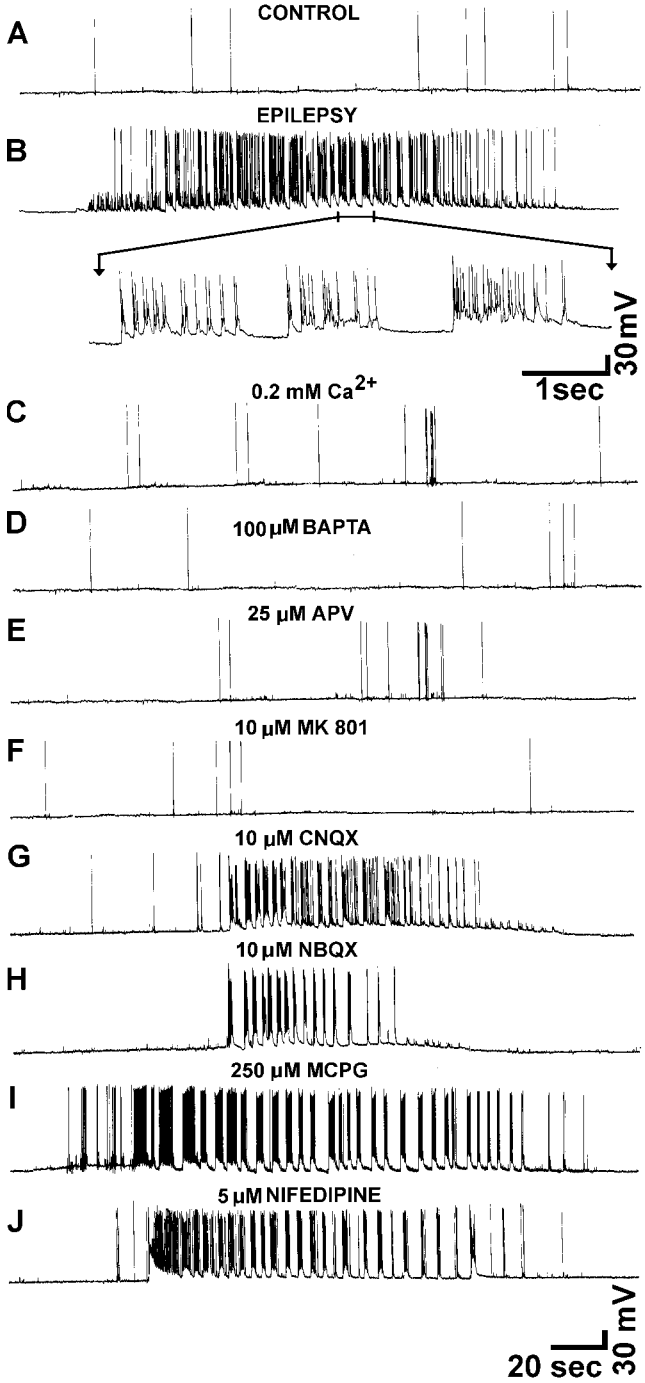

FIG. 2. Induction of epilepsy in cultured hippocampal neurons 2 days after low-Mg^{2+} treatment in the presence or absence of low Ca^{2+} (*C*) or neuropharmacological agents (*D*—*J*) during the brief 3-hr exposure to low Mg^{2+}. After low-Mg^{2+} treatment, cultures immediately were returned to normal Mg^{2+} levels in the media. Intracellular recordings were obtained from hippocampal pyramidal neurons 2 days after low-Mg^{2+} treatment. The recordings shown were representative of more than 10 independent experiments for each condition. SREDs (seizures) were observed under standard conditions, 2 mM Ca^{2+} (*B*), 10 μM CNQX (*G*), 10 μM NBQX (*H*), 250 μM MCPG (*I*), and 5 μM nifedipine (*J*). Low extracellular Ca^{2+} (0.2 mM, *C*) or intracellular chelation of Ca^{2+} with 100 μM BAPTA (*D*) during low-Mg^{2+} treatment completely blocked the development of epilepsy despite causing even a higher frequency seizure discharge during the low-Mg^{2+} treatment (Fig. 1). Expansion of a 5-sec interval of seizure activity in *B* demonstrated the numerous spikes associated with each epileptiform burst. More than 80 spike discharges occurred during this 5-sec segment, giving an average spike frequency discharge of 16 Hz during this segment of the seizure shown in *B*. The electrophysiological patterns for conditions *A*–*J* were representative of recordings taken earlier or later than the 2-day post-SE sample time. Conditions that blocked the induction of epilepsy never manifested seizure discharges.

techniques were performed on neurons before, during (Fig. 1), and 2 days after (Fig. 2) the 3-hr exposure to SE, using procedures routinely performed in our laboratory (7, 15, 16). During low-Mg^{2+} exposure, neurons developed sustained epileptiform discharges with larger and longer-duration depolarizing waves and multiple action potentials evolving into continuous tonic, high-frequency burst discharges that were equivalent to the electrographic seizure discharges observed in SE (Fig. 1*B*). Control neurons before low-Mg^{2+} treatment manifested spontaneous excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs, respectively) (7) that intermittently reached threshold and triggered individual action potentials (Fig. 1*A*). Control neurons never manifested SREDs ($n = 350$).

The low-Mg^{2+} (SE) treatment produced long-lasting plasticity changes in the neurons, resulting in the development of SREDs. This "epileptic" state persisted for 2 days after treatment (Fig. 2*B*) and for the life of the neurons in culture (7). Fig. 2*B* presents a spontaneous representative epileptiform discharge that occurred 2 days after the low-Mg^{2+} treatment. This epileptiform discharge lasted 2 min and 40 sec and was characterized by paroxysmal depolarizing shifts (PDSs) and sustained spike discharges. The SREDs self-terminated as manifested by gradually decreasing EPSPs. In various neurons sampled, SREDs ranged from 3 to 20 Hz and lasted from 10 sec to 3 min, with the majority lasting from 20 to 40 sec. The frequency of SREDs varied from one to two per minute to as few as one per hour in different cultures. Thus, prolonged seizure activity caused the neurons to develop permanent plasticity changes, leading to the development of SREDs.

To evaluate whether prolonged increase in $[Ca^{2+}]_i$ during SE was involved in causing the induction of "epilepsy", $[Ca^{2+}]_i$ was measured during SE, utilizing confocal laser-scanning microscopy with the Ca^{2+}-binding dye indo-1 (17, 18). These studies produced the first images of intracellular calcium levels before, during, and after electrographic SE-like discharges (Fig. 3*A*–*C*). This technique provided a dynamic method to quantitate $[Ca^{2+}]_i$ (Table 1) while simultaneously measuring membrane potentials by intracellular recordings (Fig. 1). During SE, $[Ca^{2+}]_i$ increased from basal levels of 150 nM to 577 nM $[Ca^{2+}]_i$ (Fig. 3 *A* and *B*; Table 1). After SE, $[Ca^{2+}]_i$ returned to near-control levels (Fig. 3*C*). Thus, SE caused a sustained increase in $[Ca^{2+}]_i$ for the duration of SE, suggesting that elevated $[Ca^{2+}]_i$ may play a role in causing the long-lasting plasticity changes involved in developing SREDs.

To further evaluate the role of Ca^{2+} in causing SREDs, we determined whether lowering Ca^{2+} in the extracellular medium during SE could block the induction of epileptogenesis. Extracellular Ca^{2+} was decreased from 2 mM to 0.2 mM during SE. Lowering extracellular Ca^{2+} blocked the SE-induced increase in $[Ca^{2+}]_i$ from 577 to 177 nM (Table 1, Fig. 3*E*) without decreasing the intensity or duration of SE (Fig. 1*C*). Thus, it was possible to prevent the increase in $[Ca^{2+}]_i$ during SE without decreasing the intensity or duration of the epileptiform discharge during the low-Mg^{2+} treatment (Table 1). The effect of reducing extracellular calcium during SE on epileptogenesis was evaluated intracellularly by using the whole-cell current–clamp recording mode 2 days after the SE treatment. Low calcium during SE blocked the development of SREDs (Fig. 2*C*). Prolonged recordings (1 hr or greater) from more than 20 neurons incubated in low Ca^{2+} during SE did not reveal a single epileptiform discharge 2 days posttreatment (Table 1). Thus, lowering of extracellular calcium was able to

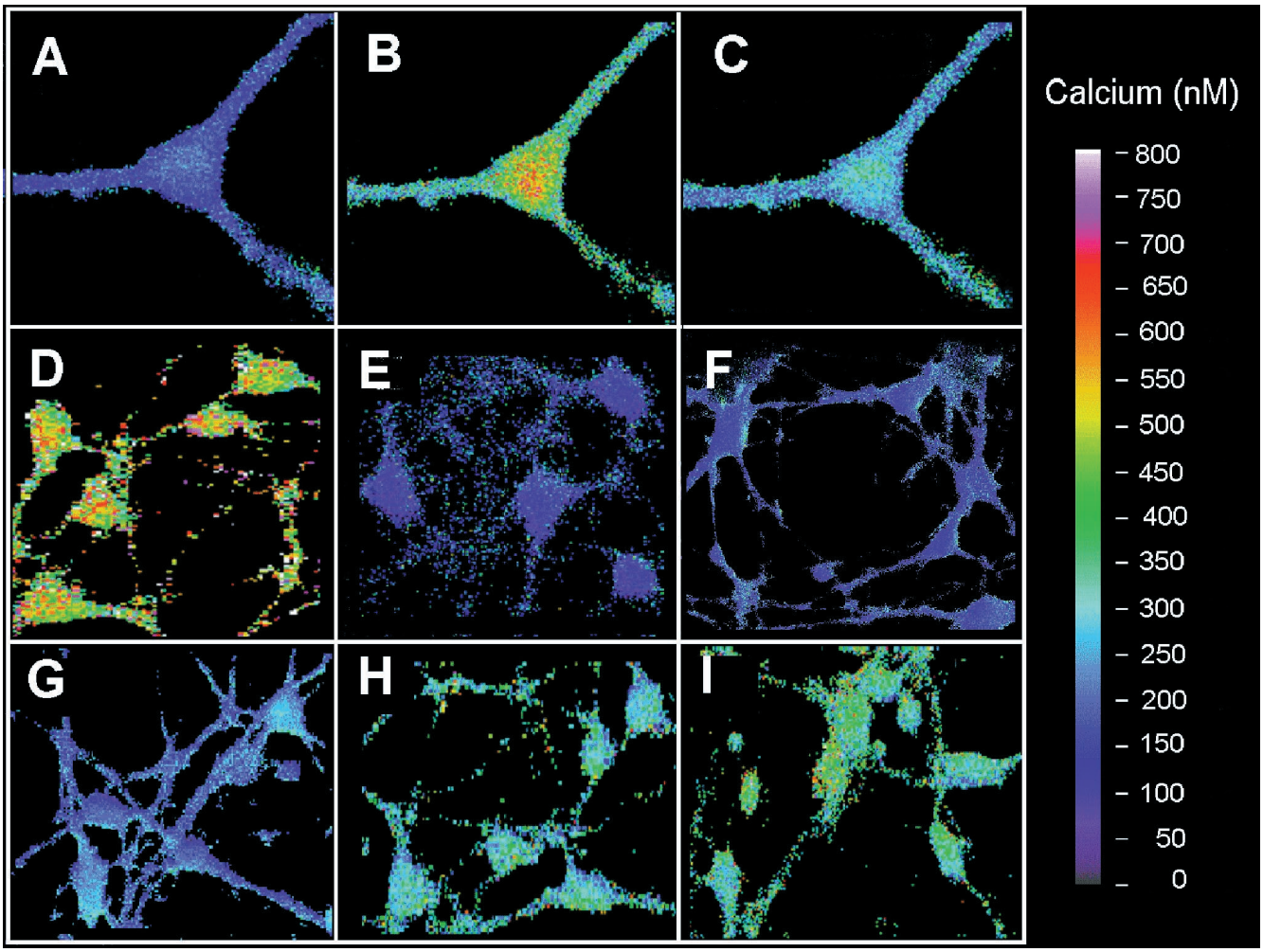

FIG. 3. $[Ca^{2+}]_i$ imaging of hippocampal neurons in culture before, during, and after low-Mg^{2+} treatment in the presence or absence of low Ca^{2+} or various neuropharmacological agents. (*A*) A representative hippocampal neuron under conditions with basal $[Ca^{2+}]_i$ levels of 150 nM. (*B*) The same neuron shown in *A* during low-Mg^{2+} treatment. $[Ca^{2+}]_i$ rose to 570 nM. (*C*) The same neuron shown in *A* and *B* after return to normal Mg^{2+} conditions after 3 hr of treatment. $[Ca^{2+}]_i$ returned to near-basal conditions. Neurons remained viable for as long as 8–12 hr during Ca^{2+}-imaging studies (7). *D–I* represent groups of neurons during low-Mg^{2+} treatment with normal (2 mM, *D*) or low Ca^{2+} (0.2 mM, *E*), or the presence of 100 μM BAPTA (*F*), 10 μM MK801 (*G*), 10 μM CNQX (*H*), and 5 μM nifedipine (*I*). The calibration bar provides a direct comparison of color intensity and $[Ca^{2+}]_i$ levels in nM.

block SE-induced increases in $[Ca^{2+}]_i$ and totally prevented the development of SREDs in this model of epilepsy.

To evaluate on epileptogenesis the effects of other techniques used to block the increases in $[Ca^{2+}]_i$ caused by SE, neurons were exposed to 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetate (BAPTA), an intracellular Ca^{2+}-chelating agent (19), during the low-Mg^{2+} treatment. BAPTA treatment did not alter the intensity (Table 1) or duration of epileptiform discharge during SE (Fig. 1*D*). However, BAPTA treatment during SE prevented the increase in $[Ca^{2+}]_i$ by chelating the Ca^{2+} after it entered the neuron, maintaining a $[Ca^{2+}]_i$ during SE that essentially was identical to resting $[Ca^{2+}]_i$ levels (Fig. 3*F* and Table 1). Chelation of $[Ca^{2+}]_i$ during SE also completely blocked the development of SREDs (Fig. 2*D*). Barium as a second messenger has been shown to be unable to substitute for calcium in many of calcium's second-messenger effects (20). Substituting barium for calcium as a divalent cation during SE also prevented the development of SREDs (data not shown). In addition, blocking synaptic transmission and preventing seizure discharges during the SE treatment with tetrodotoxin (TTX) prevented both the rise in $[Ca^{2+}]_i$ (Table 1) and the induction of SREDs in this model (data not shown). These results demonstrated that the prolonged increases in $[Ca^{2+}]_i$ during SE caused persistent plasticity changes in hippocampal neurons that induced "epilepsy."

Since these results provide direct evidence that increased $[Ca^{2+}]_i$ during SE caused SREDs, it is important to determine whether all sources of increased $[Ca^{2+}]_i$ or selective Ca^{2+} pathways were involved in inducing epileptogenesis. The increase in $[Ca^{2+}]_i$ caused by SE could be mediated by several types of calcium channels (21, 22). Activation of the NMDA receptor is a receptor-mediated mechanism to activate calcium entry. Activation of metabotropic, AMPA, and kainic acid receptors also depolarize neurons and activate voltage-gated calcium channels (VGCC) (21). Specific AMPA channel subtypes also can allow Ca^{2+} entry (21, 22). Depolarization during seizure discharge also activates L- and N-type VGCC (21, 22). To investigate the relative contributions of receptor and voltage-gated Ca^{2+} channels in the induction of epileptogenesis in this model, several specific pharmacological inhibitors of these channels were employed. These experiments evaluated the contribution of each specific mechanism for increasing $[Ca^{2+}]_i$ and for inducing SREDs.

The NMDA receptor antagonists 2-amino-5-phosphonovaleric acid (APV) and MK-801 (23, 24) both were effective in decreasing the rise in $[Ca^{2+}]_i$ during SE from 577 nM to 293 nM and 287 nM (Table 1), demonstrating that some, but not

Table 1. Quantitation of $[Ca^{2+}]_i$ before and during low-Mg^{2+} treatment (SE) and the development of "epilepsy" 2 days later in the presence or absence of various pharmacological additions

Condition	$[Ca^{2+}]_i$, nM†	Epilepsy‡	SE spike frequency§
Control	165 ± 10	No	0
Low-Mg^{2+} (SE)	577 ± 35	Yes	12 ± 4.4
+ Low Ca^{2+} (0.2 mM)	177 ± 18**	No	10 ± 3.2
+ BAPTA (100 μM)	160 ± 8**	No	14 ± 5.1
+ APV (25 μM)	293 ± 24**	No	16 ± 3.8
+MK-801 (10 μM)	287 ± 25**	No	11 ± 2.9
+CNQX (10 μM)	433 ± 10**	Yes	9 ± 3
+NBQX (10 μM)	422 ± 56**	Yes	8 ± 2.4
+Nifedipine (5 μM)	441 ± 19*	Yes	13 ± 4.1
+TTX (1 μM)	157 ± 17**	No	0

†The $[Ca^{2+}]_i$ quantitation was determined from the average $[Ca^{2+}]_i$ for individual neurons under each condition by using a Ca^{2+} calibration curve (17). The data represent the mean ± SE for each determination ($n = 10$). Each of the additions to the SE condition lowered $[Ca^{2+}]_i$ in a statistically significant manner (*, $P < 0.03$; **, $P < 0.005$; Students t test).

‡The presence or absence of spontaneous recurrent seizures (epilepsy) at 2 days after a 3-hr low-Mg^{2+} treatment under each condition was determined by intracellular whole-cell current-clamp recordings of individuals neurons ($n = 10$) for more than 10 min each to determine the presence or absence of epileptiform activity. Cells either manifested epileptiform discharges or overt seizure activity (Yes) or showed no evidence of hyperexcitability (No). The development of epileptigenesis in these preparations was "all or none" in that no transition excitability states were observed at the 2-day time point. Representative examples of epileptic discharges (*B*, *G–J*) and nonexcitable recordings (*A*, *C–F*) are shown in Fig. 2. The results demonstrate that the decrease in $[Ca^{2+}]_i$ produced by multiple treatment conditions during SE were not equally effective in inducing epileptogenesis and were specific to the NMDA receptor-activated Ca^{2+}-transduction pathway.

§The data represent the mean ± SE of spike frequency for spike discharges during low-Mg^{2+} treatment for each experimental condition ($n = 8$). Spike frequency was determined as described previously. In each condition where low Mg^{2+} produced continuous epileptiform discharges, the discharges were continuous and persistent at the same amplitude as shown in Fig. 1 for the duration of the low Mg^{2+} treatment. There were no statistical differences between the spike frequencies of these conditions (Student's t test).

all, of calcium entry during SE was through the NMDA receptor channel complex. The ability of NMDA receptor inhibition to partially block the rise in $[Ca^{2+}]_i$ during SE is shown in Fig. 3*G*. Moreover, both MK-801 and APV were able to completely block the induction of epilepsy in this model (Fig. 2 *E* and *F*). These results demonstrated that the partial reduction in $[Ca^{2+}]_i$ by selective inhibition of NMDA receptor-specific Ca^{2+} entry completely blocked the induction of SREDs.

The kainic acid and AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (25) and the more selective AMPA receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline (NBQX (25) also were effective in reducing SE-dependent increases in $[Ca^{2+}]_i$ from 577 nM to 433 nM and 422 nM (Table 1 and Fig. 3*H*). However, in contrast to NMDA receptor inhibition, this reduction in $[Ca^{2+}]_i$ produced by CNQX and NBQX had no effect on the induction of epileptogenesis (Fig. 2 *G* and *H*). Treatment with the metabotropic glutamate receptor antagonist, MCPG (26), during SE did not alter the epileptiform activity during SE (Fig. 1*I*). It also did not prevent the development of SREDs (Fig. 2*I*) or decrease the $[Ca^{2+}]_i$ increase during SE (data not shown). Inhibition of the L voltage-gated Ca^{2+} channels by nifedipine (27) was effective in reducing the overall calcium increase due to SE from 577 nM to 441 nM (Table 1; Fig. 3*I*), but had no effect in preventing the induction of epileptogenesis (Fig. 2*J*). Inhibition of the N-type voltage-gated Ca^{2+} channels with φ-conotoxin (27) during low-Mg^{2+} treatment prevented neurotransmission and blocked the development of SE. Thus, it was not possible to use this agent during SE without abolishing SE.

It is important to establish that the antagonists and agents used to evaluate the mechanisms for increasing $[Ca^{2+}]_i$ did not affect the duration or severity of SE. The effects of each of these drugs on SE were evaluated intracellularly by using whole-cell current–clamp techniques (Fig. 1 and Table 1). The spike frequency and duration of SE was not affected significantly by low calcium, BAPTA, MK-801, APV, CNQX, NBQX, or nifedipine (Table 1). By quantitating $[Ca^{2+}]_i$ during SE and electrophysiologically measuring the intensity of SE, it was possible to demonstrate directly that calcium entering through the NMDA transduction pathway was selectively responsible for the induction of SREDs in this model. The different treatments shown in Table 1 had some effect on the duration of paroxysmal depolarization shifts, but the actual number of spike discharges was not statistically different. In addition, some of the minor apparent differences in spike frequency shown graphically in Fig. 1 were a result of differences in spike frequency from one neuron to the other.

DISCUSSION

Understanding basic mechanisms that underlie epileptogenesis will play a major role in improving the treatment of epilepsy and in preventing the development of this condition (3). Results from this study provide evidence that prolonged activation of the NMDA receptor–Ca^{2+} transduction pathway can cause the development of SREDs in populations of hippocampal neurons for the life of the neurons in culture. These persistent changes in neuronal excitability also represent an important model of neuronal plasticity. Prolonged elevations of $[Ca^{2+}]_i$ in the presence of NMDA receptor inhibition did not induce epileptogenesis, demonstrating a role for the NMDA-activated Ca^{2+} transduction pathway in inducing this plasticity change. Although other molecular mechanisms may play a role in the development of epileptogenesis, our findings provide direct evidence for a molecular mechanism involving Ca^{2+} in mediating the long-term plasticity changes that lead to spontaneous recurrent epileptiform discharges that are analogous to electrographic seizures.

Recent studies from our laboratory in an *in vivo* model of partial complex epilepsy have demonstrated that the induction of epilepsy was dependent on NMDA receptor activation (28). The induction of hyperexcitability in the kindling (5) and hippocampal slice (11) models also can be inhibited by NMDA receptor antagonists. However, it is not feasible in these models of epilepsy to be able to directly quantitate NMDA receptor activated Ca^{2+} entry while simultaneously evaluating altered neuronal excitability. The results from this study provide direct evidence that prolonged NMDA receptor-activated increases in $[Ca^{2+}]_i$ during SE can cause the induction of symptomatic epilepsy and suggest that the effects of NMDA receptor inhibition in blocking epileptogenesis in intact models of epilepsy are a result of the inhibition of the NMDA–Ca^{2+} transduction pathway.

Substantial progress has been made in our understanding of how changes in $[Ca^{2+}]_i$ effect long-term physiological changes in neurons. There are multiple sites by which Ca^{2+} can enter the neuron and Ca^{2+} can activate numerous specific enzyme systems or trigger specific Ca^{2+} transduction pathways (27, 29). The specificity of entry sites, localization of the signal, subcellular activation of selective enzyme systems, and duration of the signal all combine to provide a diversity of Ca^{2+} transduction systems. The results in this paper demonstrate that prolonged activation of the NMDA–Ca^{2+} transduction pathway at nonlethal levels for 2–3 hr can produce permanent plasticity changes in neurons, resulting in SREDs.

NMDA receptor-mediated elevations in $[Ca^{2+}]_i$ were shown to induce "epilepsy." This finding is consistent with the findings from Greenberg's laboratory, which show that specific Ca^{2+} signal-transducing pathways can have selective effects on cellular function and gene-transduction systems (27, 29). Long-term changes in gene expression have been associated with the induction of epilepsy (30–34), and other long-term molecular alterations may be the underlying molecular substrates responsible for the second message effects of NMDA–Ca^{2+} entry in epilepsy. Evaluating the role of prolonged NMDA–Ca^{2+} entry in producing long-term plasticity changes such as "epilepsy" not only provides an insight into the molecular basis of epilepsy, but offers new directions for developing novel therapeutic compounds that could prevent the development or treatment of this common neurological condition.

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