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Conditions Sufficient for Nonsynaptic Epileptogenesis in the CA1 Region of Hippocampal Slices

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Bikson, Marom, Scott C. Baraban, and Dominique M. Durand. Conditions sufficient for nonsynaptic epileptogenesis in the CA1 region of hippocampal slices. *J Neurophysiol* 87: 62–71, 2002; 10.1152/jn.00196.2001. Nonsynaptic mechanisms exert a powerful influence on seizure threshold. It is well-established that nonsynaptic epileptiform activity can be induced in hippocampal slices by reducing extracellular Ca^{2+} concentration. We show here that nonsynaptic epileptiform activity can be readily induced in vitro in normal (2 mM) Ca^{2+} levels. Those conditions sufficient for nonsynaptic epileptogenesis in the CA1 region were determined by pharmacologically mimicking the effects of Ca^{2+} reduction in normal Ca^{2+} levels. Increasing neuronal excitability, by removing extracellular Mg^{2+} and increasing extracellular K^+ (6–15 mM), induced epileptiform activity that was suppressed by postsynaptic receptor antagonists [D-(−)-2-amino-5-phosphonopentanoic acid, picrotoxin, and 6,7-dinitroquinoxaline-2,3-dione] and was therefore synaptic in nature. Similarly, epileptiform activity induced when neuronal excitability was increased in the presence of K_{Ca} antagonists (verruculogen, charybdotoxin, norepinephrine, tetraethylammonium salt, and Ba^{2+}) was found to be synaptic in nature. Decreases in osmolarity also failed to induce nonsynaptic epileptiform activity in the CA1 region. However, increasing neuronal excitability (by removing extracellular Mg^{2+} and increasing extracellular K^+) in the presence of Cd^{2+}, a nonselective Ca^{2+} channel antagonist, or veratridine, a persistent sodium conductance enhancer, induced spontaneous nonsynaptic epileptiform activity in vitro. Both novel models were characterized using intracellular and ion-selective electrodes. The results of this study suggest that reducing extracellular Ca^{2+} facilitates bursting by increasing neuronal excitability and inhibiting Ca^{2+} influx, which might, in turn, enhance a persistent sodium conductance. Furthermore, these data show that nonsynaptic mechanisms can contribute to epileptiform activity in normal Ca^{2+} levels.

INTRODUCTION

Most complex focal seizures either originate in or are elaborated by the hippocampus (Jensen and Yaari 1988; Spencer et al. 1987). During a hippocampal seizure (ictal episode) large neuronal aggregates are recruited into excessive, highly synchronized discharges that last several seconds or minutes (Dichter et al. 1972). In the interseizure (interictal) period the epileptogenic focus remains active generating brief (lasting tens or hundreds of milliseconds) bursts of synchronized neuronal discharge. Both spontaneous interictal- and ictal-like epileptiform discharges can be readily induced in vitro by exposing hippocampal slices to convulsant agents or by modulation of the extracellular ionic milieu (Durand 1993). It is well-established that excitatory synaptic connections mediate the initiation and propagation of interictal epileptiform discharges (Prince and Connors 1986). However, in vitro experiments have shown that ictal epileptiform can be nonsynaptic in nature (Demir et al. 1999; Haas and Jefferys 1984; Jensen and Yaari 1988; Patrylo et al. 1994). For example, several laboratories have demonstrated the development of spontaneous ictal epileptiform activity in acute hippocampal slices when synaptic transmission is blocked via a reduction of the extracellular calcium concentration of the artificial cerebrospinal fluid solution bathing the slice (Haas and Jefferys 1984; Yaari et al. 1983). This "nonsynaptic" form of epileptiform activity closely approximates ictal seizure activity (Haas and Jefferys 1984).

Calcium is an important modulator of brain function and directly influences neurotransmitter release, neuronal excitability, calcium-sensitive potassium channel function, and sodium channel function (Hille 1992; Lancaster and Nicoll 1987; Leibowitz et al. 1986). It is unknown, however, which of these effects contributes to the generation of nonsynaptic epileptiform activity observed during perfusion with low-Ca^{2+} media. The goal of the present study was to isolate those conditions sufficient for nonsynaptic bursting by pharmacologically mimicking each of the above effects in the presence of normal extracellular Ca^{2+} levels. Here we report that increased neuronal excitability in the presence of Cd^{2+}, a nonselective Ca^{2+} channel antagonist, or veratridine, a persistent sodium conductance enhancer, induced spontaneous nonsynaptic epileptiform activity in vitro. Veratridine-induced bursting was observed without suppressing synaptic transmission. These two novel models of epileptiform activity demonstrate that nonsynaptic epileptiform activity can arise in normal extracellular Ca^{2+} concentrations and suggest a novel explanation for how nonsynaptic forms of seizure activity might arise in vivo.

METHODS

Preparation of hippocampal slices

All experiments were performed in the CA1 or CA3 pyramidal cell regions of hippocampal brain slices prepared from Sprague-Dawley

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rats (75–250 g). Rats were anesthetized using ethyl ether and decapitated. The brain was rapidly removed and one hemisphere glued to the stage of a Vibroslicer (Vibroslice, Campden Instruments, Loughborough, UK). Slicing was carried out in cold (3−4°C), oxygenated sucrose-based artificial cerebrospinal fluid (ACSF) consisting of (in mM) 220 sucrose, 3 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 26 $NaHCO_3$, 2 $CaCl_2$, and 10 dextrose. The resulting 300- to 350-μM-thick slices were immediately transferred to a holding chamber containing "normal" ACSF consisting of (in mM) 124 NaCl, 3.75 KCl, 1.25 KH_2PO_4, 2 $CaCl_2$, 2 $MgSO_4$, 26 $NaHCO_2$, and 10 dextrose, held at room temperature and bubbled with 95% O_2-5% CO_2. Slices were held at room temperature for ≥60 min before being transferred to the submerged recording chamber where they were initially perfused with oxygenated normal ACSF (temperature, 34 ± 2°C). Slices in which the CA1 pyramidal cell layer was not clearly visible were not used. A total of 202 hippocampal slices was used in this study. Results are reported as means ± SD; *n* is the number of slices.

Drugs and solutions

Low-Ca^{2+} ACSF consisted of (in mM) 124 NaCl, 4.75 KCl, 1.25 KH_2PO_4, 0.2 $CaCl_2$, 1.5 $MgSO_4$, 26 $NaHCO_3$, and 10 dextrose. Zero-Mg^{2+} ACSF consisted of (in mM) 124 NaCl, 5.75 KCl, 1.25 KH_2PO_4, 2.0 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose. As noted, in some experiments the total K^+ concentration was adjusted by omission or addition of KCl. Veratridine was dissolved in 0.1 mM HCl solution. The stock solution (0.1 mM) of veratridine (Sigma) was prepared and stored at −4°C. Picrotoxin, 5,5-diphenylhydantoin salt (phenytoin), 6,7-dinitroquinoxaline-2,3-dione (DNQX), D-(−)-2-amino-5-phosphonopentanoic acid (D-APV), 4-aminopyridine (4-AP), norepinephrine (NE), tetraethylammonium salt (TEA), $CdCl_2$, and $BaCl_2$ were obtained from Sigma. Verruculogen and charybdotoxin were obtained from Alomone. All drugs and solutions were applied for >30 min.

For current-clamp studies, patch pipettes were filled with internal recording solution consisting of (in mM) 140 KGluconate, 10 KCl, 1 $MgCl_2$, 0.025 $CaCl_2$, 2 Na-ATP, 0.2 Na-GTP, 0.2 EGTA, and 10 HEPES. All internal patch solutions were pH adjusted to 7.2 with 10 M KOH (285–295 mOsm).

Extracellular field and potassium recording

Extracellular recordings of field potentials were made using glass micropipettes (2–5 MΩ) filled with 150 mM NaCl. Single-barreled potassium-selective microelectrodes were constructed using established methods described elsewhere (Amman 1986; Ghai et al. 2000). We utilized *N*,*N*-dimethyltrimethylsilylamine (Fluka Chemicals) to silanize the electrode tips, and the Fluka 60398 potassium-selective membrane solution, which contains the potassium ionophore Valinomycin. The potassium-selective microelectrodes were filled with 150 mM KCl. Electrodes were calibrated in 0.1, 1, 10, and 100 mM KCl using the separate solution method (Amman 1986). Only electrodes of 95% Nernstian slope were used. Electrodes were ≥1,000-fold selective for potassium over sodium. Recording electrodes were positioned in the pyramidal cell layer of the CA1 region. When both a potassium-selective and field electrode were used, the electrodes were positioned within 50 μM of each other, and the potential recorded by the field electrode was subtracted from the potential recorded by the ion-selective electrode.

Signals were amplified and low-passed filtered (0.1–1 kHz) with an AxoClamp 2B or 1D amplifier (Axon Instruments), an FLA-01 amplifier (Cygnus Technology) and stored on a DAT (MicroData System). After digitization (DigiData 1200, Axon), data from potassium recordings was band-stop filtered. Monopolar stimulating electrodes were placed on the surface of the alveus (antidromic stimulation) or stratum radiatum (orthodromic stimulation). The spread of spontaneous burst propagation was determined by dividing the difference in event onset time measured by two field electrodes by the electrodes' separation distance.

Whole cell recording

Tight-seal (4−6 GΩ) current-clamp recordings were made with an Axopatch-1D amplifier (Axon Instruments). Patch pipettes were pulled from 1.5-mm borosilicate filament containing glass tubing (Warner Instrument) using a two-stage process, firepolished, and coated with silicone elastomer (Sylgard; Dow Corning). The pipette was positioned under visual control with differential interference contrast optics and infared light (IR-DIC). Unless otherwise stated, no holding current was used during current-clamp recordings. Data were transferred directly to a computer using a DigiData 1200 board and pCLAMP software (Axon Instruments). All cells were within 150 μM of the field electrode and held for >20 min.

Nonsynaptic bursting

Nonsynaptic bursting was identified based on the following criteria: *1*) the activity should approximate low-Ca^{2+} activity in burst frequency (<0.5 Hz), duration (>1 s), and propagation velocity (approximately 1 mm/s) across the CA1 pyramidal cell layer; *2*) once induced activity should remain stable for ≥2 h; *3*) activity should persist in the presence of postsynaptic receptor antagonists (D-APV, picrotoxin, DNQX); and *4*) the activity should be reproduced as reliably as low-Ca^{2+} bursting. In contrast, "synaptic" epileptiform activity originates in the CA3 region, propagates quickly across the CA1 layer (approximately 100 mm/s), and is completely suppressed when synaptic function is depressed (Prince and Connors 1986).

RESULTS

Reduction in extracellular Ca^{2+}

Consistent with previous studies (Haas and Jefferys 1984; Yaari et al. 1983), incubation of slices in low-Ca^{2+} medium resulted in the development of spontaneous nonsynaptic epileptiform activity ($n = 12$ of 18 slices tested). Both single peak ($n = 8$, Fig. 1*B*) and multiple peak ($n = 4$, Fig. 1*A*) events were observed. Low-Ca^{2+} bursting activity was not suppressed after addition of 100 μM Cd^{2+}, to block voltage-activated Ca^{2+} channels ($n = 2$, Fig. 1*B*). Low-Ca^{2+} bursts propagated slowly (<1 mm/s) across the CA1 pyramidal cell layer (Fig. 1*C*). The average low-Ca^{2+} burst frequency, amplitude, and duration were 7.3 ± 4.6 bursts/30 s, 1.1 ± 0.3 mV, and 1.9 ± 0.7 s, respectively.

Increased neuronal excitability

The effects of the reduction in extracellular Ca^{2+} concentration can be grouped into those involving increased neuronal excitability (due to a reduction in extracellular cation screening) and those resulting from a reduction in Ca^{2+} influx (intracellular Ca^{2+}). We tested whether increasing excitability, in itself, was sufficient to generate nonsynaptic epileptiform activity. In these experiments, neuronal excitability was increased by removing extracellular Mg^{2+} (Hille 1992) and raising extracellular K^+ concentration (zero-Mg^{2+} ACSF). Through charge screening, divalent cations in the extracellular space shift the voltage dependence of transmembrane ion channels as if a negative voltage bias has been added (Hille 1992). Thus the reduction in cation screening by reduction of extracellular Ca^{2+} by 1.8 mM (low-Ca^{2+} ACSF) can be mimicked, in part, by removal of extracellular Mg^{2+} ions. However,

because Ca^{2+} is a more effective charge "screener," the removal of extracellular Mg^{2+} does not excite neurons as significantly as reduction in extracellular Ca^{2+}. Therefore in some experiments, neuronal excitability was further increased by elevation of extracellular potassium.

Consistent with previous studies (Dreier and Heinemann 1991), incubation of slices in zero-Mg^{2+} ACSF resulted in the development of spontaneous inter-ictal epileptiform activity in the hippocampus (Fig. 2*A*, $n = 27$). This activity was synaptic in nature as indicated by the pacing of CA1 by CA3, the event waveform, and the short inter-burst interval. Further enhancement of neuronal excitability by incremental increases in extracellular K^+ initially enhanced (6–10 mM) and then suppressed (12–15 mM) spontaneous bursting but did not result in the development of spontaneous nonsynaptic epileptiform activity ($n = 44$).

To test the hypothesis that the presence of synaptic activity interferes with the initiation of nonsynaptic bursting, we added postsynaptic receptor antagonists. Addition of a postsynaptic receptor antagonist "cocktail" containing picrotoxin (100 μM), DNQX (40 μM), and D-APV (50 μM) suppressed zero-Mg^{2+} inter-ictal bursting but did not result in the development of nonsynaptic epileptiform activity ($n = 9$). These results indicate that increased neuronal excitability is not sufficient by itself to induce nonsynaptic bursting activity in the CA1 region of hippocampal slices.

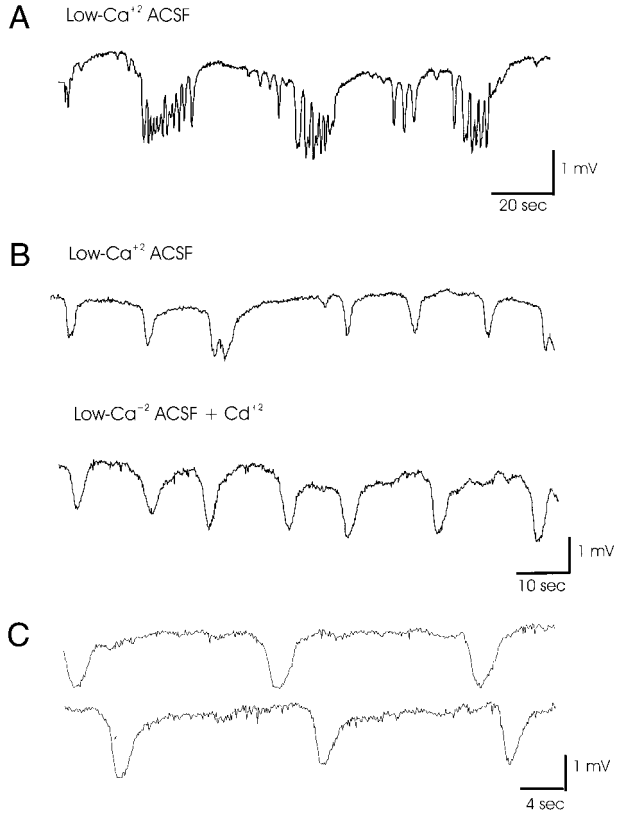

FIG. 1. Spontaneous low-Ca^{2+} ictal epileptiform activity. Both multiple peak events (*A*) and single peak activity (*B*) was observed. Effect of Cd^{2+} of low-Ca^{2+} bursting. *C*: simultaneous bursting recorded at 2 sites along CA1 pyramidal cell layer.

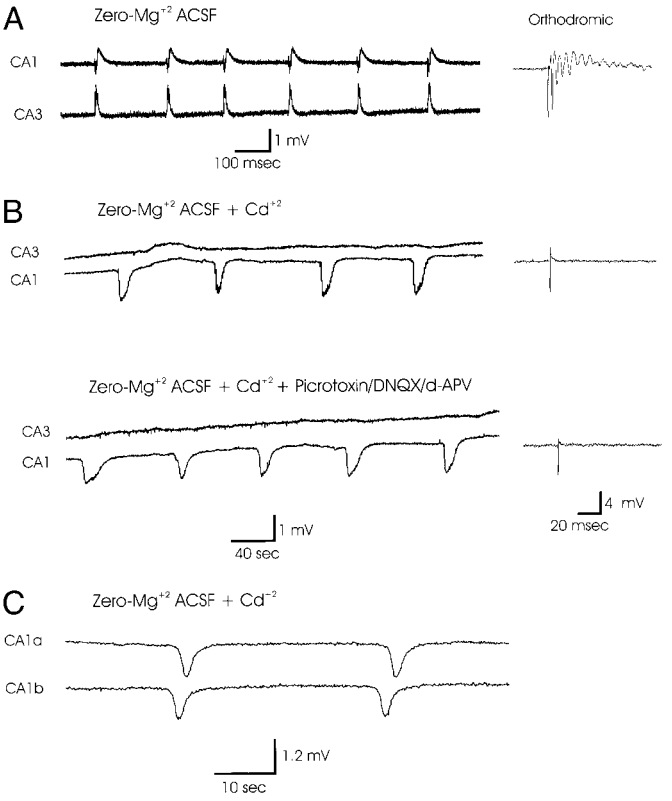

FIG. 2. Spontaneous epileptiform activity in the CA3 and CA1 and evoked orthodromic response in CA1 (*right column*) monitored with field electrodes. *A*: spontaneous inter-ictal bursting during incubation in zero-Mg^{2+} medium. *B*: spontaneous ictal epileptiform activity during perfusion with in zero-Mg^{2+} plus Cd^{2+}. Effect of postsynaptic receptor antagonists on zero-Mg^{2+} plus Cd^{2+} bursting. *C*: simultaneous bursting recorded at 2 sites along CA1 pyramidal cell layer.

Increased neuronal excitability and inhibition of Ca^{2+} influx

To test the hypothesis that both increased neuronal excitability and decreased Ca^{2+} influx are sufficient to initiate nonsynaptic bursting, voltage-activated Ca^{2+} channels were blocked with a nonselective Ca^{2+} channel antagonist Cd^{2+} (100 μM), and neuronal excitability was increased as above (by removing extracellular Mg^{2+} and elevating extracellular K^+ concentration). Addition of Cd^{2+} mimics the effect of extracellular Ca^{2+} reduction in inhibiting intracellular Ca^{2+} transients but does not mimic the increase in excitability resulting from reduced cation screening (which is reproduced by zero-Mg^{2+} ACSF, see above).

Incubation of slices in zero-Mg^{2+} ACSF with added Cd^{2+} (zero-Mg^{2+} plus Cd^{2+}) suppressed synaptic epileptiform activity and resulted in the development of nonsynaptic bursting in CA1 ($n = 38$ of 60 slices tested, Fig. 2*B*). The induced activity resembled low-Ca^{2+} activity in waveform (average zero-Mg^{2+} plus Cd^{2+} burst frequency, amplitude, and duration were 2.7 ± 1.3 bursts/30 s, 1.2 ± 0.2 mV, and 1.5 ± 0.3 s, mean ± SD) and in propagation velocity (Fig. 2*C*). The suppression of synaptic function was confirmed by the abolition of the evoked orthodromic response. Furthermore, addition of our postsynaptic receptor antagonist cocktail (picrotoxin, DNQX, D-APV) had no effect on zero-Mg^{2+} plus Cd^{2+} bursting ($n = 3$, Fig. 2*B*).

We similarly tested the hypothesis that inhibition of Ca^{2+}

influx by itself is sufficient to induce nonsynaptic bursting. Addition of Cd^{2+} to normal ACSF did not result in any spontaneous epileptiform activity ($n = 3$). Furthermore, incubation of slices in zero-Mg^{2+} plus Cd^{2+} medium modified by the reduction of extracellular K^+ (by 1–2 mM) failed to induce spontaneous activity ($n = 13$), indicating that a minimum level of increased excitability is required for nonsynaptic bursting. Taken together, our results indicate that reduction of Ca^{2+} influx *in combination* with increased neuronal excitability is sufficient to induce nonsynaptic epileptiform activity.

Increased neuronal excitability and decreased osmolarity

In the subsequent experiments, we studied the role of Ca^{2+} channel inhibition in nonsynaptic bursting. We set out to determine what action of inhibiting Ca^{2+} influx results in the generation of spontaneous nonsynaptic bursting. Extracellular osmolarity can exert a powerful influence on seizure susceptibility (Schwartzkroin et al. 1998). Changes in extracellular ion activities have also been shown to induce sustained changes in extracellular volume fraction (EVF) (Andrew et al. 1997). We therefore tested the hypothesis that incubation of slices in low-Ca^{2+} or zero-Mg^{2+} plus Cd^{2+} solutions induces nonsynaptic bursting by simultaneously increasing excitability and decreasing the EVF. In these experiments, neuronal excitability was increased by perfusing slices with zero-Mg^{2+} solution, and the EVF was decreased by diluting the perfusate by 10% ($n = 2$) or 20% ($n = 10$). Dilution enhanced spontaneous synaptic activity but did not induce nonsynaptic bursting. To determine whether synaptic activity was interfering with the generation of nonsynaptic events, postsynaptic receptor antagonists were added to the diluted medium. Addition of picrotoxin (100 μM), DNQX (40 μM), and D-APV (50 μM) suppressed synaptic bursting but did not result in the development of nonsynaptic epileptiform activity ($n = 5$). These results demonstrate that a combination of increased neuronal excitability and cell swelling are not sufficient to induce nonsynaptic epileptiform activity in the CA1 region of hippocampal slices.

Increased neuronal excitability and inhibition of Ca^{2+}-dependent K^+ conductances

Ca^{2+}-dependent K^+ conductances (K_{Ca}) are known to exert a powerful influence on neuronal excitability (Lancaster and Nicoll 1987) and have been implicated in seizure generation (Alger and Nicoll 1980). K_{Ca} conductances are suppressed by the removal of extracellular Ca^{2+} or addition of Cd^{2+} (Aoki and Baraban 2000; Lancaster and Nicoll 1987) and could thus facilitate the initiation of nonsynaptic bursting (see above). We therefore tested the hypothesis that increased neuronal excitability in combination with inhibition of specific K_{Ca} conductances could induce nonsynaptic epileptiform activity. NE and verruculogen have been shown to inhibit the late and early components of K_{Ca}, on CA1 pyramidal cells, respectively (Aoki and Baraban 2000; Lancaster and Nicoll 1987). Perfusion of slices with either zero-Mg^{2+} plus NE (5 μM) or zero-Mg^{2+} plus verruculogen (100 nM) solutions resulted in characteristic inter-ictal synaptic activity ($n = 8$). Addition of the postsynaptic receptor antagonists cocktail (picrotoxin, DNQX, D-APV) suppressed this activity but did not result in the development of spontaneous nonsynaptic bursting ($n = 4$). Charybdotoxin has also been shown to inhibit the early component of K_{Ca} (Lancaster and Nicoll 1987). Addition of a cocktail containing charybdotoxin (40 nM), verruculogen (90 mM), NE (10 μM), and postsynaptic receptor antagonists (as above) to zero-Mg^{2+} solution did not result in spontaneous bursting ($n = 3$). Furthermore, addition of the nonspecific K^+ channel antagonists TEA (25 mM, $n = 2$) and Ba^{2+} (300–700 μM, $n = 2$) similarly failed to induce nonsynaptic bursting even after addition of postsynaptic receptor antagonists. Taken together the results indicate that reduction of Ca^{2+}-dependent K^+ conductances in combination with increased neuronal excitability is not sufficient to induce nonsynaptic epileptiform activity.

Increased neuronal excitability and enhancement of the persistent sodium conductance

The persistent sodium current is made up of "late" openings of sodium channels that continue to occur many milliseconds to seconds after the beginning of a depolarization of membrane voltage. The persistent sodium conductance has been shown to modulate the bursting characteristics of CA1 pyramidal cells (Azzouz et al. 1997) and is enhanced by reductions in extracellular Ca^{2+} (Alkadhi and Tian 1996; Azzouz et al. 1996; Leibowitz et al. 1986). We therefore tested the hypothesis that increased neuronal excitability in combination with enhancement of the persistent sodium conductance is sufficient to induce nonsynaptic epileptiform activity. Veratridine has been shown to enhance the persistent sodium conductance without increasing the fast transient sodium current (Leibowitz et al. 1986; Tian et al. 1995). Addition of veratridine (300 nM) to normal ACSF did not induce any spontaneous population bursting ($n = 2$, Fig. 3*A*). Perfusion of slices with zero-Mg^{2+} ACSF with added veratridine (zero-Mg^{2+} ACSF veratridine), however, resulted in the development of spontaneous epileptiform activity ($n = 23$ of 30 slices tested). Consistent with data from low-Ca^{2+} studies (Haas and Jefferys 1984), both fast (>100 mm/s, Fig. 3*B*) and slow (<1 mm/s, Fig. 3*C*) propagation was observed in different slices bathed in zero-Mg^{2+} plus veratridine solution. No correlation between initial propagation velocity and slice age, or slice viability (as measured by the size of the orthodromic population spike) was observed. The average burst frequency, amplitude, and duration were 2.1 $\pm$ 0.8 bursts/60 s, 1.3 $\pm$ 0.32 mV, and 24 $\pm$ 6.1 s, respectively. Importantly, the addition of veratridine did not block synaptic transmission as indicated by the persistence of the evoked orthodromic response. Addition of the postsynaptic receptor antagonist cocktail (picrotoxin, DNQX, D-APV) suppressed the orthodromic response and decreased the average propagation velocity ($n = 3$). However, with the exception of a decrease in event rise time, burst waveform was not affected by the addition of postsynaptic receptor antagonists (Fig. 3*B*).

These results suggest that increased neuronal excitability in combination with enhanced persistent sodium conductance is sufficient to induce epileptiform activity in vitro. Although this spontaneous activity persists in the presence of synaptic transmission (which can modulate its propagation velocity), it does not appear to require postsynaptic activation of neurotransmitter receptors. Furthermore, its characteristic waveform and slow propagation velocity also indicate that zero-Mg^{2+} ACSF

plus veratridine epileptiform activity is nonsynaptic in nature. However, a role for neuromodulators such as ATP and cholinergic agonists cannot be ruled out.

Pharmacology of nonsynaptic epileptiform activity

The low-Ca^{2+}, zero-Mg^{2+} plus Cd^{2+}, and zero-Mg^{2+} plus veratridine epilepsy models may arise from similar mechanisms or could represent fundamentally different forms of nonsynaptic epileptiform activity. The following sections address this question and further characterize each of these models. The role of the persistent sodium conductance in each model was tested using phenytoin. Phenytoin is a common anti-convulsant known to inhibit the persistent sodium conductance (Rogawski 1998) without decreasing normal neuronal excitability (Yaari et al. 1986). Phenytoin (50–200 μM) reversibly suppressed low-Ca^{2+} ($n = 3$), zero-Mg^{2+} plus Cd^{2+} ($n = 5$), and zero-Mg^{2+} plus veratridine ($n = 4$) induced bursting (Fig. 4). As indicated by the size of the antidromic population spike, phenytoin slightly enhanced neuronal excitability during suppression of low-Ca^{2+} activity and did not effect excitability during suppression of zero-Mg^{2+} plus Cd^{2+}-induced activity. As indicated by the size of the orthodromic population spike, phenytoin slightly enhanced either neuronal excitability or synaptic function during suppression of zero-Mg^{2+} plus veratridine epileptiform activity. These results suggest that the persistent sodium conductance plays a critical role in the generation of spontaneous epileptiform activity in each of these models.

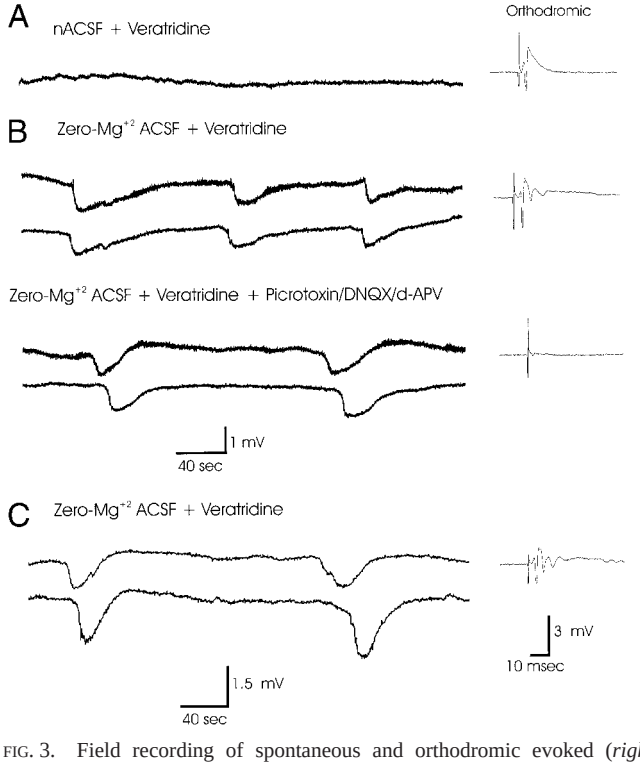

FIG. 3. Field recording of spontaneous and orthodromic evoked (*right column*) activity in CA1 pyramidal cell layer during perfusion with veratridine solution. *A*: no spontaneous activity was observed when veratridine was added to normal artificial cerebrospinal fluid (ACSF). *B*: simultaneous recording from 2 sites along CA1 pyramidal cell layer of fast propagating bursting during perfusion with zero-Mg^{2+} plus veratridine solution. Effect of postsynaptic receptor antagonists on fast propagating zero-Mg^{2+} plus veratridine bursting. *C*: in a different slice, simultaneous recording from 2 sites along CA1 pyramidal cell layer of slow propagating bursting during perfusion with zero-Mg^{2+} plus veratridine solution.

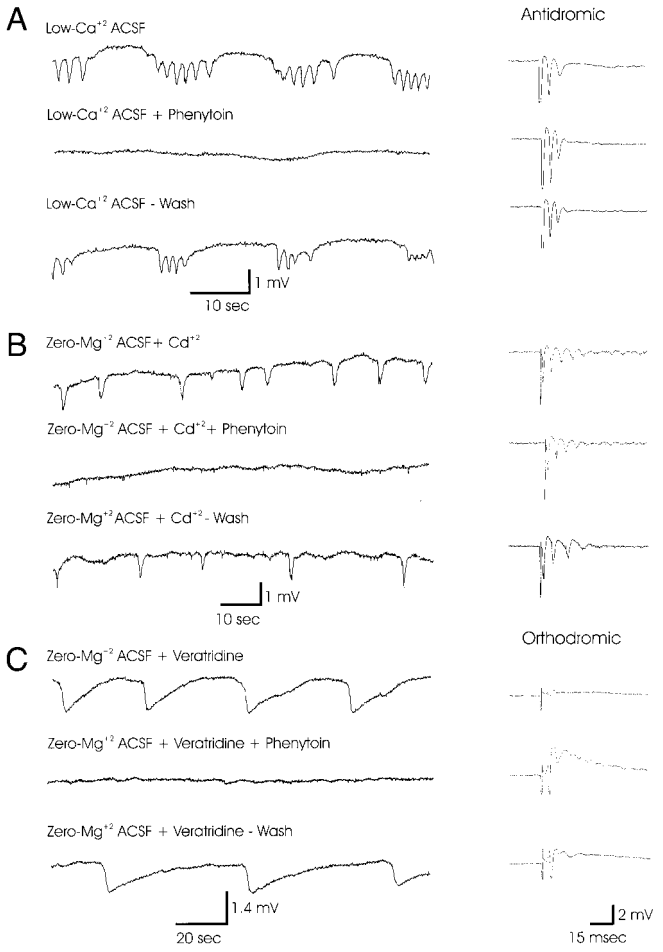

FIG. 4. Effect of phenytoin on spontaneous low-Ca^{2+} (*A*), zero-Mg^{2+} plus Cd^{2+} (*B*), and zero-Mg^{2+} plus veratridine nonsynaptic epileptiform activity (*C*). Phenytoin reversibly suppressed each activity. In each case an antidromic or orthodromic evoked response was monitored in CA1 (*right column*).

Potassium transients associated with nonsynaptic epileptiform activity

Previous studies have shown that low-Ca^{2+} epileptiform activity is always associated with a transient increase in extracellular potassium (Yaari et al. 1983). This potassium "wave" has been shown to facilitate the nonsynaptic propagation of epileptiform activity (Lian et al. 2000). Furthermore, the clearance of potassium by glia through spatial buffering has been suggested to contribute to the generation of low-Ca^{2+} burst field shifts (Bikson et al. 1999; Yaari et al. 1983). We used ion-selective electrodes to study the potassium transients associated with each of these models. Consistent with data from in vitro interface recording environments, low-Ca^{2+} epileptiform activity always correlated with a transient increase in extracellular potassium (Fig. 5*A*, $n = 3$). Similar transients were observed during zero-Mg^{2+} plus Cd^{2+} (Fig. 5*B*, $n = 5$) and zero-Mg^{2+} plus veratridine (Fig. 5*C*, $n = 4$) epileptiform activity. In all cases, the waveform and amplitude of the recorded potassium transients was highly correlated with that of the field shift, suggesting a glial spatial buffering role for each model.

Intracellular recordings during epileptiform activity

CA1 pyramidal cells were patched during spontaneous epileptiform activity to monitor the intracellular activities associated with each type of nonsynaptic bursting. Consistent with data from previous studies (Haas and Jefferys 1984), during perfusion with low-Ca^{2+} solution, pyramidal cells depolarized to -52 ± 6.3 mV and began firing tonically ($n = 5$). At this resting membrane potential (RMP), action potentials were grouped individually, in doublets, or in triplets (Fig. 6*A*). In each case, low-Ca^{2+} field bursts were associated with a further depolarization of approximately 5–10 mV and an increase in spontaneous action potential rate. During perfusion with zero-Mg^{2+} plus Cd^{2+} solution, pyramidal cells depolarized to -51 ± 2.0 mV and began firing spontaneous action potentials ($n = 5$). During each zero-Mg^{2+} plus Cd^{2+} field burst, cells either slightly depolarized and increased their firing rate (Fig. 6*B, left, n* = 2) or fired a robust burst of action potentials on a large (approximately 20 mV) plateau potential (Fig. 6*B, right, n* = 3). Each zero-Mg^{2+} plus veratridine field burst was associated with a large intracellular burst ($n = 3$, Fig. 6*C*). The intracellular burst always initiated with a sharp increase in membrane potential (approximately 20 mV) that decayed slowly to baseline (-41 ± 8.4 mV). Action potentials were only observed at the initiation of the intracellular burst.

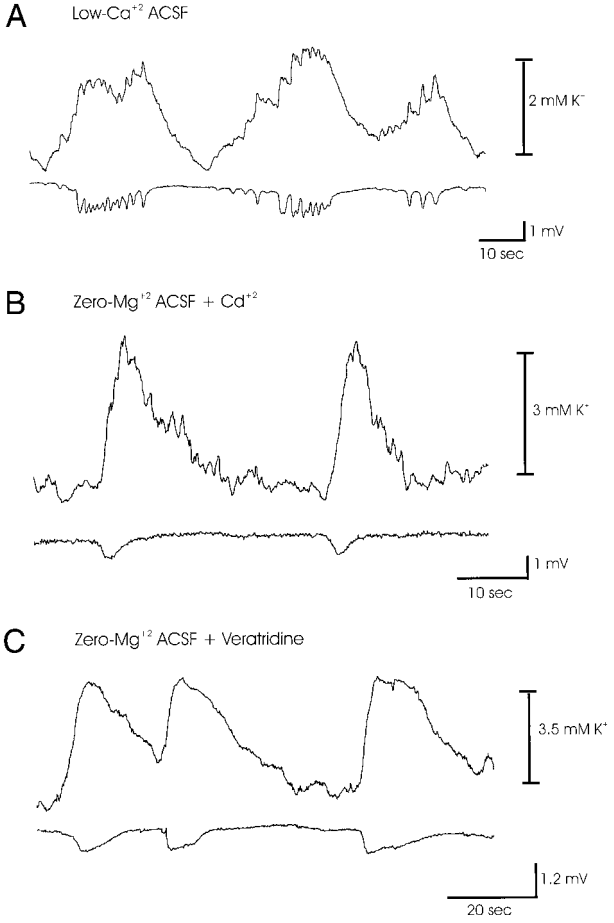

FIG. 5. Simultaneous field and potassium measurements in CA1 during spontaneous low-Ca^{2+} (*A*), zero-Mg^{2+} plus Cd^{2+} (*B*), and zero-Mg^{2+} plus veratridine bursting (*C*).

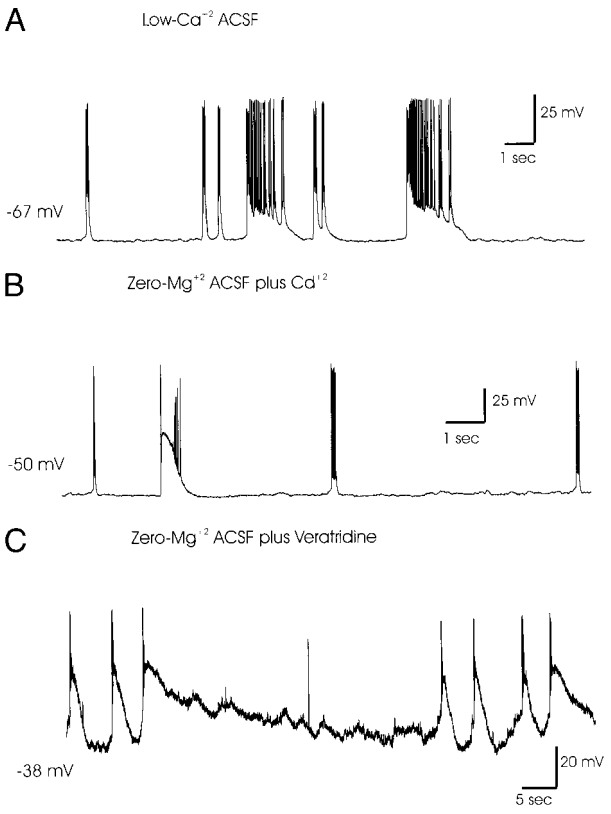

FIG. 6. Intracellular measurements in CA1 during spontaneous low-Ca^{2+} (*A*), zero-Mg^{2+} plus Cd^{2+} (*B*), and zero-Mg^{2+} plus veratridine bursting (*C*).

Spontaneous intracellular bursts

During perfusion with low-Ca^{2+} solution, in the absence of field burst activity, CA1 pyramidal cells, at RMP, fired spontaneous action potentials grouped in singlets, doublets, and triplets. Cells exhibiting all three types of behavior could be induced to fire longer spontaneous intracellular bursts (SIBs) by hyperpolarization of the membrane by approximately 10–20 mV (Fig. 7*A*, $n = 4$). Hyperpolarization-induced bursts were characterized by a large approximately 25-mV depolarization that was sustained 0.5–2 s. In the presence of field bursts, SIBs were often, but not necessarily, triggered by field events. During perfusion with zero-Mg^{2+} plus Cd^{2+} medium, in the absence of field bursts, a majority of cells either exhibited spontaneous bursts similar to low-Ca^{2+}–induced SIBs at RMP, or could be induced to fire spontaneous bursts by slight hyperpolarization of the membrane (Fig. 7*B*, $n = 3$). During perfusion with zero-Mg^{2+} plus veratridine solution, in the absence of synchronized field bursts, CA1 pyramidal cells always fired prolonged SIBs. Veratridine-induced SIBs were similar in duration (approximately 5–10 s) to intracellular bursts observed during field burst activity and were similarly characterized by action potential generation at the onset of bursting activity followed by a prolonged plateau potential during which action potential generation was generally suppressed (Fig. 7*C*, $n = 3$). The veratridine-induced SIBs were occasionally interrupted by a sustained depolarization (approximately 10 mV) lasting 20–60 s, during which spontaneous activity was suppressed. Similar, phenytoin-sensitive, veratridine-induced SIBs have previously been reported in normal ACSF (Otoom and Alkadhi 2000; Tian et al. 1995).

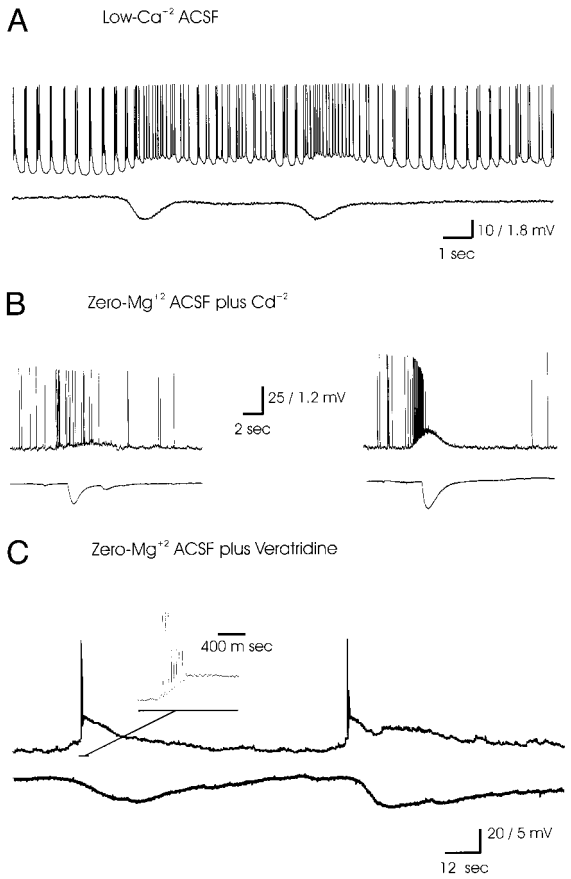

FIG. 7. Intracellular recording from CA1 pyramidal cells during perfusion with low-Ca^{2+} (*A*) zero-Mg^{2+} plus Cd^{2+} (*B*), and zero-Mg^{2+} plus veratridine solutions (*C*) in the absence of spontaneous field bursting.

DISCUSSION

Since the discovery that spontaneous nonsynaptic bursts could be generated in the hippocampus, there has been a great deal of interest in determining the underlying mechanisms (Jefferys 1995). Here we report that perfusion of slices in zero-Mg^{2+} plus Cd^{2+} and zero-Mg^{2+} plus veratridine solution results in the development of spontaneous nonsynaptic epileptiform activity in the CA1 region. These results demonstrate, for the first time, that nonsynaptic epileptiform activity can be induced in normal Ca^{2+} levels and with intact synaptic function. A unique feature of the zero-Mg^{2+} plus veratridine model is that it involves the *enhancement* (rather than inhibition) of a specific ion channel function.

Are low-Ca^{2+} and zero-Mg^{2+} plus Cd^{2+} bursting facilitated by an enhanced persistent sodium conductance?

Antagonists of the persistent sodium current (including phenytoin, carbamazepine, and valproate) suppress spontaneous low-Ca^{2+} epileptiform activity (Fig. 4*A*) (Heinemann et al. 1985). Reduction in extracellular Ca^{2+} has been shown to enhance the persistent Na^+ conductance and modulate the intrinsic bursting behavior of CA1 pyramidal cells (Alkadhi and Tian 1996; Azzouz et al. 1996; Xiong et al. 1997). Enhancement of this current could thus facilitate low-Ca^{2+} bursting. There are several mechanisms by which a reduction in extracellular Ca^{2+} can enhance sodium-mediated inward rectification. First, a reduction in extracellular Ca^{2+} results in decreased cation screening, which shifts the voltage dependence of all membrane-bound ion channels, including the persistent sodium, as if a tonic depolarizing bias has been added (Hille 1992). Second, a reduction in extracellular Ca^{2+} can also facilitate sodium influx through Ca^{2+} channels (Hablitz et al. 1986). Third, extracellular Ca^{2+} reduction can facilitate Na^+ influx through a nonselective ion channel (Xiong et al. 1997).

It has been shown using intracellular Ca^{2+} imaging that the onset of low-Ca^{2+} bursting coincides with intracellular Ca^{2+} depletion (Takiyama et al. 1998). The slow onset of low-Ca^{2+} and zero-Mg^{2+} plus Cd^{2+} activity is consistent with a gradual wash out of intracellular Ca^{2+} stores. The enhancement of the persistent sodium current by reductions in extracellular Ca^{2+} follows a similar slow time course (Azzouz et al. 1996). Low-Ca^{2+} bursts are accompanied by transient decreases in extracellular Na^+ (Yaari et al. 1983). Last, computer simulations indicate that enhancement of the persistent sodium conductance underlies the generation of bursting behavior of CA1 pyramidal cells exposed to low-Ca^{2+} solution (J. W. Shuai, personal communication).

Interestingly, each of the nonsynaptic in vitro models we studied was associated with similar but distinct intracellular activities. These distinctions are, perhaps, the strongest evidence suggesting that each model could represent a different type of nonsynaptic bursting (with different conditions contributing to spontaneous burst generation). This paradigm would predict that low-Ca^{2+} and zero-Mg^{2+} plus Cd^{2+} solutions induced spontaneous nonsynaptic bursting by inhibiting a Ca^{2+}-dependent process(es) not tested in this study. In addition, neuromodulators such as ATP and cholinergic agonists could play a role in zero-Mg^{2+} plus veratridine bursting.

Nonsynaptic nature of ictal epileptiform activity

Previous studies have demonstrated the development of both ictal and inter-ictal epileptiform activity in CA1 when neuronal excitability is increased by raising extracellular potassium concentration (Jensen and Yaari 1988). However, in high-K^+ solution, ictal activity does not occur in 2 mM Ca^{2+}, but only when extracellular Ca^{2+} is reduced to 1.2 mM (Leschinger et al. 1993). Furthermore, ictal bursts generated in high-K^+ are associated with a further drop in extracellular Ca^{2+} of $\leq$0.4 mM. Similarly, Patrylo et al. (1994) showed that in the dentate gyrus, extracellular Ca^{2+} reduction to 0.9 mM was required to induce high-K^+ bursting at physiological ranges. High-K^+ (reduced Ca^{2+}) ictal, but not inter-ictal bursting, in both CA1 and the dentate gyrus, has been shown to be nonsynaptic in nature (Jensen and Yaari 1988; Patrylo et al. 1994).

Perfusion of slices in reduced Mg^{2+} ACSF can induce ictal activity in the entorhinal cortex. However, this ictal activity is not stable over time and does not invade the CA1 region (Dreier and Heinemann 1991). Similarly, 4-aminopyridine (4-AP), an A-type potassium channel blocker, can induce ictal bursting in the entorhinal cortex but not in the CA1 region (Bruckner and Heinemann 2000). The nonsynaptic nature of 4-AP and reduced Mg^{2+} ictal activity have not been studied in detail; however, they exhibit a distinct pharmacology from inter-ictal bursting. For example, in both these models, ictal, but not interictal, events are suppressed by phenytoin (Bruck-

ner and Heinemann 2000; Zhang et al. 1995). Ictal bursting generated in the CA1 region using the low-$C1^-$ model has been shown to be nonsynaptic (Demir et al. 1999). Last, in the present study, both the novel zero-Mg^{2+} plus Cd^{2+} and zero-Mg^{2+} plus veratridine models were shown not to depend on synaptic transmission.

While previously it has been suggested that ictal bursting is facilitated by an increase in synaptically driven inter-ictal activity, numerous slice studies have shown that inter-ictal epileptiform activity inhibits ictal burst generation in the high K^+ (Jensen and Yaari 1988), zero-Mg^{2+} (Bragdon et al. 1992; Swartzwelder et al. 1987), and 4-AP (Barbarosie and Avoli 1997) models. Similarly, in intact animal models (Engel and Ackerman 1980; Gotman 1984) and human studies (Engel et al. 1981; Gotman and Marciani 1985) interictal electroencephalographic (EEG) spiking does not always correlate with seizure frequency or severity. The absence of interictal activity during low-Ca^{2+}, zero-Mg^{2+} plus Cd^{2+}, and zero-Mg^{2+} plus veratridine bursting is consistent with these findings.

Both seizures and ictal epileptiform events are always associated with extracellular potassium transients (Dietzel et al. 1989). Furthermore, these potassium transients are thought to underlie the generation of the electrographic signal itself (Dietzel et al. 1989). The slow waveform, propagation velocity, and frequency of electrographic seizures and ictal bursting are consistent with potassium dynamics known to underlie nonsynaptic bursting (Bikson et al. 1999; Lian et al. 2000; Yaari et al. 1983). While synaptic mechanisms have been shown to underlie the paroxysmal depolarizing shift of inter-ictal activity (Prince and Connors 1986), it has yet to be shown how physiological synaptic function can produce relatively slow and irregular ictal events. Last, in the hippocampus, ictal activity originates almost exclusively in the CA1 regions (Haas and Jefferys 1984; Jensen and Yaari 1988; Patrylo et al. 1994), where the tight packing of cell bodies promotes nonsynaptic interactions, such as extracellular potassium build-up (Jefferys 1995). In contrast, inter-ictal activity always originates in the CA3 region where recurrent synaptic connections are abundant (Prince and Connors 1986).

Traditionally, nonsynaptic bursting was thought only to occur in the low-Ca^{2+} and zero-Ca^{2+} models (Jefferys 1995). Taken together, previous findings and the results presented here suggest that a large component of in vitro ictal epileptiform activity could be nonsynaptic in nature. Furthermore, because the tightly packed cell bodies of the hippocampal CA1 region provide ideal conditions for nonsynaptic interactions such as ephaptic and electrotonic interactions and extracellular ionic fluctuations (Jefferys 1995), the propensity of the hippocampus to be a seizure focus in vivo could be a result of its predisposition to nonsynaptic epileptiform activity.

Sodium channel defects, relevance to seizures in vivo

It has been reported that an increase in the number of sodium channels in the neurons of genetically seizure-susceptible E1 mice is responsible for their predisposition to epileptic seizures (Sashihara et al. 1992). An increase in the conductance of individual Na^+ channels was also found in another epileptic animal model, the tottering mouse (Willow et al. 1986). Altered levels of sodium channel subunits I, II, and III (Bartolomei et al. 1997) and sodium channel β2 subunit (Gastaldi et al. 1998) mRNA were found in the hippocampus of kainate-treated epileptic rats. Kearny et al. (2001) recently reported that in a transgenic mouse model (designated GALQ3), a mutation *enhancing* the persistent sodium current in hippocampal pyramidal neurons, led to the development of focal hippocampal seizures. Thus in mice, altered sodium channel function can play a critical role in seizure genesis.

Similarly in humans, several sodium channel mutations have been linked with epileptic phenotypes including a point mutation of the sodium channel β1 subunit gene located on chromosome 19q13.1 (Wallace et al. 1998). The human β1 subunit *prolongs* neuronal depolarization when co-expressed in vitro with a rat brain sodium channel α subunit RBII. Point mutations in the voltage-sensor regions of the sodium channel α-subunit SCN1A have been associated with seizures in two families (Escayg et al. 2000). Another candidate gene for epilepsy, the SCN5A sodium channel (previously thought to be expressed only in heart tissue) is selectively expressed in only the limbic system of the brain (Hartmann et al. 1999). In the heart, several mutations of this channel have been described that *prolong* sodium currents and result in prolonged QT syndrome and cardiac arrythmia. Furthermore, the relative expression ratio of sodium channel subtype I to subtype II is increased in the brain of epileptic patients (Lombardo et al. 1992).

Last, the persistent sodium current is a well characterized pharmacological target for controlling seizures. Most clinically useful anti-convulsants with known mechanisms of action (including valproic acid, phenytoin, carbamazepine, and lamotrigine) reduce sodium currents (Bialer et al. 1999; Rogawski 1998). Furthermore, in almost all in vitro epilepsy models, phenytoin effectively suppresses ictal, but not synaptic inter-ictal bursting (see above). Despite increasing evidence associating genetic Na^+ mutations with seizure susceptibility and a long-standing anticonvulsant pharmacology exploiting sodium channel function, there has been little insight into how a specific Na^+ channel defect could give rise to seizure activity. The results of the present study suggest that alteration of a persistent sodium channel is a critical component of nonsynaptic epileptiform bursting.

Conclusions

The results of this study suggest several mechanisms by which a specific channel mutation could promote the development of nonsynaptic seizures in vivo and that the generation of nonsynaptic seizures in vivo would not require a reduction in extracellular Ca^{2+} or an interruption of normal synaptic function. Furthermore, these results show that nonsynaptic interactions are sufficient in themselves to generate epileptiform activity in the presence of normal Ca^{2+} levels and intact synaptic function and thus emphasize the critical role nonsynaptic mechanisms play in all types of seizure genesis. The two novel nonsynaptic models of epileptiform activity developed in this study provide powerful new tools toward determining the specific contribution of these mechanisms.

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