Interneuron and Pyramidal Cell Interplay During In Vitro Seizure-Like Events

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Introduction

Epileptic seizures are often described as “hypersynchronous” events arising from decreased inhibition with excessive excitation (Penfield and Jasper 1954; Westbrook 1991). However, inhibition is retained in samples from human epileptics (Babb et al. 1989; During and Spencer 1993; Menendez de la Prida et al. 2002) and interneuron activity is essential for the generation of in vitro epileptiform activity in human (Cohen et al. 2002) and animal (Avoli et al. 1996a; Fujiwara-Tsukamoto et al. 2004; Higashima et al. 1996; Lopantsev and Avoli 1998; Perez-Velazquez and Carlen 1999) tissue. Additionally, EEG (Schiff et al. 2005) and in vitro synaptic currents (Netoff and Schiff 2002) suggest that seizures may not be characterized by a straightforward synchrony increase.

Previous in vitro studies of intracellular excitatory–inhibitory (EI) interplay focused primarily on stimulation-induced epileptiform activity (Fujiwara-Tsukamoto et al. 2004, 2005; Perez-Velazquez 2003; Perez-Velazquez and Carlen 1999). In these paradigms, tetanic stimulation replaced preictal and ictal onset dynamics and the resulting afterdischarges demonstrated synchronous activation of inhibitory and excitatory cells (Fujiwara-Tsukamoto et al. 2004). However, we know little about EI interactions and synchrony during spontaneous preictal, ictal, and postictal events.

To investigate EI, excitatory–excitatory (EE), and inhibitory–inhibitory (II) interactions in robust seizure-like events (SLEs), we used a 4-aminopyridine (4-AP) and decreased magnesium seizure model. Simultaneous dual and triple whole cell recordings of inhibitory oriens interneurons and excitatory pyramidal cells, along with extracellular potential measurements, were performed in the CA1 of rat hippocampal slices. We report a novel pattern of EI activity interplay during these spontaneous SLEs.

Methods

Animals

Experiments were performed on Sprague-Dawley rats (P18–P35) with a George Mason University approved protocol.

Electrophysiology

Animals were anesthetized with diethyl-ether and decapitated, brains were removed, hippocampi isolated, and transverse 350 μm sections cut in cold dissection buffer (in mM: 2.6 KCl, 1.23 NaH2PO4, 24 NaHCO3, 0.1 CaCl2, 2 MgCl2, 205 sucrose, 20 glucose) using a vibratome.

Slices were incubated for 1 h in artificial cerebrospinal fluid (ACSF; pH 7.3, 30°C, in mM: 130 NaCl, 0.6 MgSO4, 3.5 KCl, 1.2 CaCl2, 10 glucose, 2.5 NaH2PO4, 24 NaHCO3) aerated with 95% O2-5% CO2 transferred to a submersion recording chamber (Warner Instruments), and perfused (1 ml/min, 34°C). Borosilicate glass micropipettes (4–7 MΩ) for current-clamp recordings contained (in mM): 116 K gluconate, 6 KCl, 0.1 CaCl2, 2 MgCl2, 205 sucrose, 20 glucose) using a vibratome.

Alignment of the seizure start times was based on an identifiable feature of the extracellular recording—the fast positive extracellular shift (FPES, 1–5 mV, Fig. 1D). Oriens interneurons (see Supplementary Materials1) were targeted because of their importance and vulnerability in epilepsy (Oliva et al. 2002; Sanon et al. 2005; Santhakumar and Soltész 2004) and their role in network synchronization (Gillies et al. 2002; Pike et al. 2000). Seven of 12 interneurons that underwent depolarization block (DB) displayed oriens-lacunosum

1 The Supplementary Material for this article (2 figures and additional text) is available online at http://jn.physiology.org/cgi/content/full/01378-2005/DC1.

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molecular (O-LM) interneuron membrane characteristics and bipolar horizontal dendritic morphology (Maccarreri and Lacaille 2003; Maccaferri and McBain 1996; see Supplementary materials). Cells selected for patching were 30–200 μm apart (average 122 μm). An extracellular electrode was placed in the pyramidal near the patched cells. A subset of experiments was performed after isolating CA1 (n = 13) by making cuts from alveus to molecular lacunosa on each side of CA1. Recordings were performed using Axoclamp 2B and 2A, MCC 700 (Molecular Devices), and Grass P16 amplifiers, filtered (5 kHz whole cell, 1 kHz extracellular) and digitized at 10 kHz (Digidata and Pclamp7, Molecular Devices).

SLEs appeared after 100 μM bath application of 4-AP (Sigma) and raising the temperature to 36°C. Increasing concentrations of 4-AP were used (≤200 μM) if SLE formation was not observed within 20 min. We fortuitously discovered that decreasing the Mg concentration to 0.6 mM, although insufficient by itself to promote SLEs, had a profound effect on prolonging 4-AP-induced SLEs. 4-AP increases inhibitory and excitatory signaling (Avoli et al. 1988, 1996b; Somjen 2004), whereas decreased Mg partially removes N-methyl-D-aspartate (NMDA) receptor blockade and divalent charge screening. A similar strategy uses elevated potassium with decreased magnesium (Isaev et al. 2005; Khosravani et al. 2003, 2005).

Histochemistry

Neurobiotin-filled cells were reconstructed with camera lucida (Fig. 2A and Supplementary material). For the cells that exhibited DB, outlines of the slices and stratum pyramidale were drawn using camera lucida and overlaid (to the best fit) in Adobe Photoshop (Fig. 2E).

Correlation analysis

To track the temporal evolution of EE and EI synchrony, two unbiased crosscorrelations were calculated using subthreshold membrane potentials and spike times of pairs of neurons over discrete time windows. We use crosscorrelation to infer the presence of synchronization (Netoff and Schiff 2002). Dual-correlation analyses allowed us to compare synchrony in subthreshold inputs and suprathreshold neuronal outputs. Alternate methods to achieve a similar analysis used median filters to remove spikes (Buonomano 2003; Mooney et al. 2001), which alter the spectra and are less suitable for correlation measures.

For spike correlation, a simple threshold was used to identify discrete spike times. To measure subthreshold correlations, data between spike initiation and termination were discarded and correlations were performed using interspike data (Fig. 1, A and B). The spike data gaps were retained, leaving the time relationships among the residual subthreshold voltages unchanged. This subthreshold activity reflects not only synaptic network inputs to the cells but also intrinsic voltage-gated subthreshold responses. Furthermore, the correlation variance and time length. Only statistically significant correlations, greater than 2 SDs of the expected value, were included in the analysis (full details in Supplementary materials).

Statistical analysis

Averaged data representing spiking rates and crosscorrelation values are reported as mean ± SD. ANOVA was used to determine whether the means of correlation measures from distinct SLE epochs were significantly different. Post hoc Tukey multiple-comparison tests (confidence limit P < 0.001) were performed after ANOVA. Because the number of SLEs measured per cell pair was variable (5–18), we also recalculated averages by randomly resampling to equalize the
number of SLEs compared from each pair. Both pooled and resampled averages are reported.

RESULTS

Recurrent SLEs (>15 s ictal-like network events with extracellular potential shifts; Fig. 1, A and D) were recorded in 30 neuronal pairs (18 dual and four triple) located in strata oriens and pyramidale, yielding over 250 SLEs for analysis. Qualitatively analogous SLEs were observed in experiments in which the CA1 region was surgically isolated (n = 13 cells), suggesting that the excitatory Schaeffer collateral pathway is not necessary for local network SLE generation in the CA1 (Barbarosie and Avoli 1997; Netoff and Schiff 2002).

Pyramidal cells (n = 35) fired at lower rates than interneurons before SLEs. Consistent early features of SLEs were a burst of pyramidal cell spikes (about 1–3 s), coinciding with the FPES in the extracellular and intracellular potentials followed by a sustained negative extracellular shift (1–5 mV, Fig. 1D). When multiple FPESs were seen, the final one just before the slow negative extracellular potential shift was selected as the temporal anchor point for subsequent analysis.

Sub- and suprathreshold crosscorrelation analyses of excitatory cell pairs (n = 15) revealed qualitatively different results (Fig. 1, E and F). Pyramidal cell spike output increased considerably during the SLEs (n = 15 pairs, 148 SLEs) and showed a substantial increase in spike crosscorrelation (Fig. 1E). In contrast, averaged subthreshold EE correlations (n = 15 pairs; 148 SLEs) showed significantly decreased correlations at the start of the ictal-like event (Fig. 1F). This finding is consistent with previous results in which voltage-activated conductances during similar recordings were blocked (Netoff and Schiff 2002).

In the presence of 4-AP, oriens interneurons were more active and fired at higher frequencies than concurrently recorded pyramidal cells (Figs. 2 and 3). During SLEs, the interneurons and pyramidal cells exhibited a robust spiking interplay (Fig. 2), which took place over substantially longer timescales compared with the interplay seen in interictal bursts (see Supplementary Fig. 1).

Interneuron firing rates peaked at 62 Hz just before entering into the long-lasting (5–40 s) DB within the ictal discharge. DB was characterized by transmembrane potential depolarization (to −40 to −10 mV) and the inactivation of action potential–generating mechanisms (Bikson et al. 2003; Bragin et al. 1997; Somjen 2004). DB was observed in 12 horizontal interneurons located in stratum oriens and the pyramidale–oriens layer border (Fig. 2E). Depolarization block was rarely pronounced in pyramidal cells and, when observed, was significantly shorter (<2 s) than in interneurons (Fig. 2D). As seen in Figs. 2 and 3, pyramidal cells produced sustained barages of spikes during interneuron DB, typically firing 6–15 spikes/s (average 8.28 spikes for the first 5 s of the ictal event). Note the fortuitous extracellular single-unit recording from the axon of the interneuron in Fig. 2, C and D, confirming cessation of inhibitory spiking during DB. SLE termination was accompanied by the return of interneuron activity as pyramidal cells became progressively quiescent.

Figure 3 shows an example of simultaneous EE and EI interactions during a triple whole cell and extracellular recording of a spontaneous SLE and the respective average (n = 8
SLEs) firing rates. This example illustrates that the firing rates of the two recorded pyramidal cells changed together, with peak firing rates at the onset and sustained high firing rates during the interneuron DB.

Analysis of 102 SLEs from EI pairs is shown in Fig. 3, C–E (n = 12 pairs). There was an increase in EI subthreshold correlations at the beginning and toward the end of the interneuron DB. With resampling, the increase in subthreshold correlations toward the end of the SLEs was the most robust feature (Fig. 3C).

A significant increase in EI spike correlations was observed at the beginning (unweighted; about 45 s, Fig. 3D). These EI subthreshold and spike findings are consistent with the fact that pyramidal cells and interneurons were active together at the onset and offset of the depolarization block.

The spike rate averages (n = 12 pairs, 102 SLEs) in Fig. 3E show a consistent robust sequence, starting with intense interneuron firing, followed by a peak in pyramidal cell firing during interneuron DB, and concluding with the return of intense inhibitory firing as pyramidal activity decays. The terminal decay in pyramidal cell firing frequency is associated with the second peak and sustained firing in interneurons recovering from the block (about 35 s, Fig. 3E and inset).

Figure 4 shows an example of two interneurons participating in a SLE. Although spiking activity ceased as both interneurons entered DB, subthreshold correlation values nevertheless increased (n = 13 SLEs, three pairs). The finding that pyramidal cell spike correlations are increased during interneuron DB (Fig. 3C) is consistent with synchronous excitation contributing to the subthreshold II correlations during their DB. In contrast, the loss of inhibitory spike outputs is consistent with the decrease in EE subthreshold correlations during DB (Fig. 1F).

**DISCUSSION**

Our fundamental finding is the novel interleaved spiking pattern between pyramidal cells and interneurons during spontaneous in vitro hippocampal seizure-like events. Our analysis of spike output between EI pairs revealed that pyramidal cells and interneurons became activated at different times during SLEs. Orien interneurons were more active at SLE onset, followed by a period of DB during which pyramidal cells exhibited runaway excitation. SLE termination was characterized by the oriens interneurons emerging from DB into progressively more active spiking and the simultaneous gradual reduction of pyramidal cell spiking.
Neuronal interplay and depolarization block may have been first observed in pioneering in vivo studies of single intracellular neocortical and hippocampal seizure recordings from untyped cells (Kandel and Spencer 1961; Matsumoto and Marsan 1964). More recently, a strong inhibitory component of seizures with increased inhibitory spiking before and after paroxysmal discharges was observed in single intracellular in vivo recordings from fast-spiking putative interneurons (Timofeev et al. 2002). Additional recent in vivo observations have clearly shown that precisely interleaved activity of subpopulations of inhibitory cells coordinate normal hippocampal spike synchrony (Klausberger et al. 2003). Examples of in vitro EI interplay have been observed in spontaneous bursts with elevated K⁺ (Aradi and Maccaferri 2004). We have also observed similar activity in our preparation (see Supplementary Fig. 1) and have observed that the spontaneous SLEs in our solution retained features of the burst EI interplay, but at the much longer timescale of seizures. This is in contrast to tetanically stimulated seizure-like afterdischarges that show an apparent EI synchronization (Fujiwara-Tsukamoto et al. 2004; Perez-Velazquez and Carlen 1999). We are aware of no other observations of such EI interactions during spontaneous SLEs.

In our work we explicitly calculated the correlation between distinct neuronal subtypes. We found that correlations during SLEs depended qualitatively on whether subthreshold or spike information was considered. During the SLE onset, we observed a decrease in the subthreshold input correlation among EE pairs. This finding is consistent with our previous work in which we pharmacologically suppressed active conductances (Netoff et al. 2002). EE spike output correlation, on the other hand, increased during SLEs. Similar increases in spike correlation were observed in vivo between nearby putative neocortical pyramidal cells (Timofeev et al. 2002).

It remains to be explained how hippocampal pyramidal cells, with sparse associational connections, exhibit prominent spike synchronizations in the setting of decreased subthreshold correlations. It is possible that such spikes synchronize through their ephaptic electric fields (Traub 1985a,b) or through gap junction connectivity between the axons of pyramidal cells (Draguhn et al. 1998). These effects are likely to be more effective when interneurons coordinate their DB activity by their own gap junctions (Zhang et al. 2004), thus concurrently releasing the pyramidal cells from inhibition. Indeed, our preliminary evidence suggests that the gap junction blocker carbenoxolone in our preparation blocks SLEs and, at low concentrations, shortens DB in interneurons (see Supplementary Fig. 2).

In contrast to EE, EI correlation was elevated at the beginning and end of SLEs. These findings are consistent with the fact that EI spike synchrony can occur only when both populations of cells are firing—at the beginning and toward the end of inhibitory DB. The loss of inhibitory spikes during DB is coincident with the simultaneous decrease in pyramidal cell subthreshold EE synchronization. Finally, subthreshold correlation increased while the interneurons in DB stopped firing and pyramidal cell spike correlation increased. We suggest that correlated EE spiking during interneuron DB delivers synchronous synaptic currents to interneurons at a time during which they are unable to respond by spiking.

Nonetheless, we are cautious in evaluating data after spike extraction. The residual subthreshold time series is likely undersampled synaptic currents that are coincident with spike generation, potentially leading to a spurious decrease in subthreshold EE correlation. However, our results are consistent with earlier findings during active conductance blockade (Netoff et al. 2002). It may be possible that overestimation of correlation resulting from the presence of active membrane conductances and underestimation through time series undersampling after spike extraction may have partially offset each other.

Although synchrony has often been implicated in seizure generation (Westbrook 1991), we are cautious in our interpretation of the observed correlation patterns in our experiments. Despite consistent patterns of correlation changes in the SLEs, we cannot infer a causal role for synchrony in orchestrating the neuronal interplay observed. Furthermore, it remains unknown what synchronization and neuronal interplay patterns will be observed in other in vitro and endogenous in vivo seizures when similarly examined.

Previous work has shown that after stimulation, chloride loading inside pyramidal cells causes inhibition to become excitatory (Fujiwara-Tsukamoto et al. 2004, 2005). It is possible that excessive inhibitory activity at the start of our SLEs became excitatory in its effects on pyramidal cells. Preliminary voltage-clamp observations (Ziburkus et al. 2005) suggest that not only pyramidal cells but interneurons as well may have elevated intracellular chloride in this model.

Most of our recorded interneurons resembled O-LM cells in their membrane properties (Fig. 2A) and horizontally oriented dendritic projections (Fig. 2B). O-LM cells also constitute the overwhelming majority of the oriens interneurons (Freund and Buzsáki 1996; Somogyi and Klausberger 2005). Nevertheless, we recognize that we likely included interneurons other than O-LM cells in our sampling.
Although seizures have traditionally been treated as monolithic entities—ictal versus nonictal—there is little to support this notion. Our findings suggest that the interplay of cellular activity supports a natural partitioning of these in vitro SLEs into stages. The motif of excessive inhibition, subsequent inhibitory breakdown through DB, and the concurrent increase in excitatory output could underlie some of the structured patterns seen in EEGs during seizures. In recent work, discrimination of human scalp and intracranially recorded seizures into at least three dynamical stages was shown (Schiff et al. 2005). The experimental findings in this present report illustrate a set of cellular interactions that underlie the evolution of stages in these in vitro SLEs. It will remain a challenge to identify the relevant cellular mechanisms that underlie the evolution of endogenous seizure stages in vivo. Finally, if inhibitory and excitatory cell types fire at different times during seizures, then our findings suggest several novel therapeutic targets for seizure control, including blocking excessive inhibitory network activity and preventing depolarization block.

ACKNOWLEDGMENTS

We are grateful to G. Buzsáki, R. Traub, T. Netoff, and S. L. Weinstein for helpful discussions.

GRANTS

This work was supported by National Institutes of Health Grants R01-MH-50006 and K02-MH-01493 to S. J. Schiff and K25-MH-01963 to E. Barreto, and a grant from the Epilepsy Foundation to J. Ziburkus.

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