Changes in action potential features during focal seizure discharges in the entorhinal cortex of the in vitro isolated guinea pig brain

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Submitted 14 March 2011; accepted in final form 9 June 2011

Trombin F, Gnatkovsky V, de Curtis M. Changes in action potential features during focal seizure discharges in the entorhinal cortex of the in vitro isolated guinea pig brain. J Neurophysiol 106: 1411–1423, 2011. First published June 15, 2011; doi:10.1152/jn.00207.2011.—Temporal lobe seizures in humans correlate with stereotyped electrophysiological patterns that can be reproduced in animal models to study the cellular and network changes responsible for ictogenesis. Seizure-like discharges that mimic seizure patterns in humans were induced in the entorhinal cortex of the in vitro isolated guinea pig brain by 3-min arterial applications of the GABA_A receptor antagonist bicuculline. The onset of seizure is characterized by a paradoxical interruption of firing for several seconds in principal neurons coupled with enhanced interneuronal firing and increased extracellular potassium (Gnatkovsky et al. 2008). The evolution of action potential features from firing break to excessive and synchronous activity associated with the progression of seizure itself is analyzed here. We utilized phase plot analysis to characterize action potential features of entorhinal cortex neurons in different phases of a seizure. Compared with preictal action potentials, resumed spikes in layer II–III neurons (n = 17) during the early phase of the seizure-like discharge displayed 1) depolarized threshold, 2) lower peak amplitude, 3) depolarized voltage of repolarization and 4) decelerated depolarizing phase, and 5) spike doublettes. Action potentials in deep-layer principal cells (n = 8) during seizure did not show the marked feature changes observed in superficial layer neurons. Action potential reappearance correlated with an increase in extracellular potassium. High-threshold, slow-action potentials similar to those observed in the irregular firing phase of a seizure were reproduced in layer II–III neurons by direct cortical application of a highly concentrated potassium solution (12–24 mM). We propose that the generation of possibly nonsomatic action potentials by increased extracellular potassium represents a crucial step toward reestablish firing after an initial depression in an acute model of temporal lobe seizures. Resumed firing reengages principal neurons into seizure discharge and promotes the transition toward the synchronized burst firing that characterizes the late phase of a seizure.

THE STUDY OF SEIZURE GENERATION (ictogenesis) is one of research priorities recognized by the international epilepsy research community (Baulac and Pitkanen 2009). A better understanding of seizure initiation and progression will possibly lead to new strategies to cure seizures resistant to available treatments. Focal seizure patterns recorded with intracranial electrodes (Engel 1993) during presurgical studies aimed at defining the boundaries of the epileptogenic region in pharmacoresistant patients demonstrated that seizure onset is often characterized by an abrupt waning of the background activity coupled with the emergence of fast rhythms in the β/γ range (for review, see de Curtis and Gnatkovsky 2009). This pattern is followed by a period of irregular activity that becomes progressively larger in amplitude and more synchronous. Within seconds, the discharges organize in wide bursts regularly spaced that precede seizure ending and postictal depression.

A similar progression of events was observed in chronic animal models of focal epilepsy (Bragin et al. 1999; Kharitishvili et al. 2006; Williams et al. 2009; Kadam et al. 2010) and in models of seizures developed in vitro (Lopantsev and Avoli 1998; Avoli et al. 2006; for review, see de Curtis and Gnatkovsky 2009). We utilized an acute model of limbic lobe seizures developed on the in vitro-isolated brain of adult guinea pigs to reproduce the focal electrographic seizure pattern observed in humans (Uva et al. 2005). Transient and partial (30–40%) disinhibition induced in this preparation by a 3-min arterial infusion of the GABA_A receptor antagonist bicuculline methiodide promoted focal seizures in the hippocampal-para-hippocampal region. These events were characterized at onset by fast activity at 20–30 Hz, sequentially followed by irregular firing and rhythmic bursting (Gnatkovsky et al. 2008). In the same study, we showed that in the medial entorhinal cortex (EC) the fast activity observed at seizure onset was generated by enhanced synchronization of inhibitory networks, mediated by intense firing of putative interneurons, and correlated with the complete disruption of neuronal firing in principal neurons (Table 1). Similar enhancement in interneuronal firing and reduction of excitation ahead of seizures was also reported in other in vitro studies performed on either hippocampal slices (Dzhala and Staley 2003; Ziburkus et al. 2006; Fujisawa-Tsukamoto et al. 2007) or in toto hippocampal/EC preparations from immature rats (Derchansky et al. 2008).

How this paradoxical blockade of firing in principal cells associated with strong interneuron firing at seizure onset can develop into the hypersynchronous activation of the bursting phase is an open question. On the basis of intracellular recordings coupled with extracellular potassium ([K+]o) measurements, we hypothesized that the reappearance of neuronal firing in principal neurons could be accounted for by two synergistic effects produced by the [K+]o elevation: 1) a reduction of the efficacy of GABAergic inhibition due to a depolarization of the GABA_A receptor-mediated reversal potential (Thompson and Gahwiler 1989) and 2) the induction of regenerative action potentials (APs) in principal cells. The latter hypothesis was verified in the present study by performing phase plot analysis of AP features in different populations of neurons during seizure progression.
Table 1.  Average values of $V_{rest}$, $V_{thres}$, $V_{max}$, and $V_{repolt}$ and depolarizing and repolarizing AP slopes (see Fig. 1) of principal neurons of the superficial EC (n = 17) and deep layers (n = 7)

<table>
<thead>
<tr>
<th>Neurons of superficial EC layers</th>
<th>Preictal</th>
<th>1st AP</th>
<th>2nd AP</th>
<th>Bursting Onset</th>
<th>Late Bursting</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{rest}$, mV</td>
<td>6.7 ± 1.1</td>
<td>18.6 ± 2.4**</td>
<td>20.2 ± 3.1</td>
<td>20.6 ± 2.8****</td>
<td>14.9 ± 1.6**</td>
<td>5.1 ± 0.7</td>
</tr>
<tr>
<td>$V_{thres}$, mV</td>
<td>19.7 ± 2.7</td>
<td>21.5 ± 1.7</td>
<td>31.1 ± 2.2</td>
<td>24.3 ± 1.5</td>
<td>21.8 ± 1.8</td>
<td>23.8 ± 2.1</td>
</tr>
<tr>
<td>$V_{max}$, mV</td>
<td>69.0 ± 2.9</td>
<td>62.2 ± 2.1</td>
<td>59.3 ± 1.8</td>
<td>63.8 ± 1.9</td>
<td>70.9 ± 2.5</td>
<td>71.7 ± 3.2</td>
</tr>
<tr>
<td>Depolarizing slope, mV/ms</td>
<td>20.2 ± 1.5</td>
<td>13.5 ± 1.1***</td>
<td>5.6 ± 0.4</td>
<td>10.0 ± 0.8***</td>
<td>12.9 ± 1.4***</td>
<td>18 ± 1.9</td>
</tr>
<tr>
<td>$V_{repolt}$, mV</td>
<td>22.1 ± 2.6</td>
<td>25.9 ± 2.2</td>
<td>34.5 ± 2.0</td>
<td>31.7 ± 1.7***</td>
<td>29.6 ± 2.8*</td>
<td>24.6 ± 1.7</td>
</tr>
<tr>
<td>Repolarizing slope, mV/ms</td>
<td>−6.4 ± 0.6</td>
<td>−4.6 ± 0.4**</td>
<td>−2.3 ± 0.3</td>
<td>−3.3 ± 0.3***</td>
<td>−4.4 ± 0.6**</td>
<td>−5.4 ± 0.3</td>
</tr>
</tbody>
</table>

Data measured with references to action potential (AP) recorded before the onset of arterial perfusion of bicuculline are expressed as means ± SE. $V_{rest}$, resting membrane potential; $V_{max}$, maximal voltage peak of the AP; $V_{repolt}$, repolarization potential; EC, entorhinal cortex. *P < 0.05, **P < 0.01, ***P < 0.001.

Phase plot analysis allows a simple evaluation of the membrane potential change value (dV/dt) vs. voltage during AP generation (Jennerick 1963; Bean 2007; Naundorf et al. 2006). The analysis of threshold potential, maximum peak amplitude, rise, and decay kinetics of APs indirectly gives information about the excitability changes of the network involved in the seizure. The data collected from a population of neurons of the superficial and deep layers of the medial EC will help to better understand the mechanisms of ictogenesis in our experimental model.

The findings were preliminarily reported in abstract form (de Curtis et al. 2010).

METHODS

The method to isolate the guinea pig brain in vitro has been described extensively (de Curtis et al. 1991, 1998; Muhlethaler et al. 2001). To measure potassium signals, we used a high-input impedance differential amplifier (Biomedical Engineering, Thornwood, NY).

To reproduce atypical nonsomatic AP firing, we performed local intra-EC injection of highly concentrated K+ solution (12–24 mM). The effective concentration of [K+]o, that reliably induced a spontaneous discharge in the tissue was slightly higher than that measured by ion-sensitive electrodes during an ictal event. This was possibly due to the fact that buffering of potassium [K+]o is different during seizures and in the artificial condition determined by local extracellular injection. The extracellular recording electrode, the ion-sensitive electrode, and the pipette for microinjection were placed within 1 mm in EC superficial layers. Brief (100–300 ms) K+ puffs were delivered with a <10-μm tip pipette using a Picospritzer II (Parker Instrumentation). Local field potential was also recorded from the K+-injecting pipette.

To characterize and compare neuronal firing during seizure progression, quantitative evaluation of dynamic changes of AP features was performed by phase plot analysis (Jennerick 1963; Bean 2007). As illustrated in Fig. 1, changes of the membrane potential with time (dV/dt) measured as mV/ms; y-axis in Fig. 1, bottom) are plotted against the instantaneous membrane potential (measured as mV; x-axes of Fig. 1). A single AP is represented as a loop in which the starting point represents the threshold membrane potential ($V_{thres}$), and the extreme right peak is the maximal voltage amplitude ($V_{max}$); the upper and lower parts of the loop describe the depolarization and repolarization phases (slopes), respectively. A software routine was stored with the ELPHO software. Off-line analysis was performed with specific LabView tools developed ad hoc by Dr. Vadym Gnatkovsky in our laboratory.
activity at 25–30 Hz (fa in Fig. 2B) coupled to a slow negative potential; the second and third phases were characterized by irregular firing and rhythmic bursting, respectively, that precede seizure termination. The intracellular firing correlates of these phases are analyzed in the following paragraphs. The cortical depth and the morphology of recorded cells are summarized in Table 2.

AP changes during seizure in neurons of superficial EC layers. Simultaneous field potential and intracellular recordings (Fig. 2A) from principal neurons of the superficial EC layers (II and III) showed that the fast activity phase correlates with a break of AP firing, coupled with sequences of small amplitude inhibitory postsynaptic potentials (IPSPs; Fig. 2B, bottom left; see also Gnatkovsky et al. 2008). During the irregular firing phase, intracellular activity was characterized by uneven AP firing and by the appearance of spike doublettes, typical of the transition toward burst firing (Fig. 2B, bottom middle). In the bursting phase, strong and coherent bursts of APs followed the extracellular activity (Fig. 2B, bottom right) until complete recovery of spontaneous firing.

Figures 3 and 4 illustrate the typical sequence of AP changes recorded in principal neurons of the superficial EC layers, examined with phase plot analysis. Average data in a population of superficial EC cells (n = 17) are illustrated in Fig. 5 and in Table 1. Compared with preictal spontaneous APs (Figs. 3Ba and 4A), at the onset of the irregular firing phase, APs diminished in amplitude (Vmax decreased), whereas Vthres showed a depolarizing shift (Figs. 3Bb and 4A). In this phase, AP depolarizing and repolarizing phases were significantly slowed down (P < 0.001 and P < 0.01 respectively). The Vrepol was shifted toward depolarized values. In the following few seconds, spike doublettes appeared (Fig. 3Bc and asterisks in Fig. 4A). The first AP of the pair within a doublette maintained the features described above, with little but not significant reduction in rising phase and repolarization and unchanged Vmax. The second AP was evidently smaller in amplitude, started at higher Vthres, and displayed a slower kinetic of repolarization (inner loops marked by arrows in Figs. 3Bc and 4). In 15 out of 17 superficial neurons, the depolarizing phase of second APs during the irregular firing phase showed two clearly separate components (arrows in Fig. 4B). During the transition to the bursting phase, the first and second APs of each burst showed features similar to APs described in the irregular firing phase (Fig. 5). During the late bursting phase Vmax and Vthres of the first AP of each burst gradually returned to values similar to a preictal AP, and depolarizing/repolarizing slopes became faster compared with the irregular firing phase (Fig. 3Bd and 5). A complete reestablishment of preictal AP features was demonstrated in all neurons (Figs. 3Be, 4A, and 5). The progression of AP changes during the transition from the fast activity phase to the irregular firing phase is described in the overlapping phase plot graphs shown in Fig. 4A. AP feature changes in a layer III neuron are illustrated in detail in Fig. 4B.

Values of Vrest, Vthres, Vmax, and Vrepol and the slope of the depolarizing and repolarizing phases that characterize AP features were quantified for the 17 superficial EC neurons, and the data are reported in Fig. 5 and Table 1. Mean values calculated for the second APs of both AP doublettes and bursts are represented by white symbols in Fig. 5. These population data demonstrate that, compared with the preictal period,
during the irregular firing and the initial bursting phases, \( V_{\text{rest}} \), \( V_{\text{thres}} \), \( V_{\text{repol}} \), \( V_{\text{max}} \), depolarized, and depolarizing-repolarizing slopes decelerated. All of these parameters recovered either during the late bursting phase or at the end of the seizure (left values in all plots of Fig. 5). Larger differences were observed in the second APs of both spike doublette and burst. In particular, \( V_{\text{thres}} \) and \( V_{\text{repol}} \) further depolarized (\( P < 0.01 \) for the irregular firing phase and \( P < 0.001 \) for bursting phase), and the slope of depolarization and repolarization further slowed down (\( P < 0.001 \) in all cases) compared with the first APs of a pair. The rising phase was characterized by a hump indicating a biphasic phenomenon starting at membrane values corresponding to the \( V_{\text{repol}} \) of the conditioning AP. This suggests the extrasomatic origin of the second spike, which does not follow the classical kinetic of depolarization and repolarization, as seen in the other seizure phases. The doublettes were preserved through the first seconds of the bursting phase; after that the membrane potential (\( V_{\text{rest}} \)) repolarized, and the APs regained their original features [see Fig. 3B (recovery)].

Table 2. Synoptic table of cells recorded in superficial and deep layers of the medial EC

<table>
<thead>
<tr>
<th>Layer</th>
<th>Depth of Cell, ( \mu m )</th>
<th>Cell Morphology</th>
<th>No. of Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>200–350</td>
<td>Pyramidal-like</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stellate</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>350–500</td>
<td>Pyramidal-like</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stellate</td>
<td>2</td>
</tr>
<tr>
<td>V</td>
<td>600–1000</td>
<td>Multipolar</td>
<td>8</td>
</tr>
</tbody>
</table>

The depth of cellular soma was calculated from the surface. Cell morphology was evaluated by histological reconstruction after intracellular biocytin injection.

Fig. 2. AP firing during a seizure induced in the entorhinal cortex (EC) of the isolated guinea pig brain by a 3-min perfusion of 50 \( \mu M \) bicuculline. A: extracellular (top trace) and intracellular (bottom trace) activity recorded in a pyramidal-like type neuron of the EC superficial layers. The arrows mark the time of onset of the seizure. The dotted line marks the resting membrane potential (rmp; \( -64.9 \pm 1.451 \text{ mV} \)). B: instantaneous AP frequency during seizure progression is shown. The seizure is subdivided in 3 typical phases: fast activity (fa) phase, irregular firing phase, and bursting phase. Samples of intracellular traces recorded during the 3 phases are illustrated under the corresponding event. Note the absence of firing and the presence of low-amplitude oscillations during the fa phase. C: the positions of the intracellular and extracellular recording electrodes in the medial part of the EC are illustrated on the left. The histological identification of the recorded cell in layer II of the medial EC is shown on the right at \( \times 20 \) and \( \times 40 \) magnifications. Calibration bar, 250 \( \mu m \).
Fig. 3. A: changes in AP features during a seizure intracellularly recorded from a pyramidal neuron of the EC superficial layers. The 3 phases of the seizure are marked. A thionine counterstaining section of the mEC for the identification of the cortex layers and the enlargement of the cell body marked with biocytin is shown. Calibration bar, 250 μm. B: examples of APs expanded from the time points marked by the square boxes in A. The relative phase plots are illustrated on the right of each AP sample. APs before the seizure (a), at the onset of the irregular firing phase (b), at a later time point in the same phase during the generation of spike doublets (c), during the bursting phase (d), and 20 min after the seizure (e) are illustrated. Rmp = −62 mV.
were analyzed by phase plot graphs and compared with control preictal and recovered APs (Fig. 6B). Spike doublettes did not appear in deep neurons during either the irregular firing phase or any other seizure phase. Population analysis (Fig. 7 and Table 2) showed a slight depolarization of $V_{\text{rest}}$, $V_{\text{thres}}$, and $V_{\text{repol}}$ and a decrease in $V_{\text{max}}$ less pronounced than in superficial neurons. The kinetic of depolarization and repolarization was not substantially modified during seizures in deep-layer neurons (not shown).

$[K^+]_o$ and AP changes. As previously demonstrated, $[K^+]_o$ increases at seizure onset, reaches a plateau during the irregular firing and bursting phases, and recovers at the end of seizure (Gnatkovsky et al. 2008). $[K^+]_o$ increased from basal values of $3.4 \pm 0.32$ mM to $7.7 \pm 4.5$ mM. The analysis of $[K^+]_o$ and $V_{\text{rest}}$, $V_{\text{thres}}$, and $V_{\text{max}}$ values during seizures (Fig. 8B) demonstrated a correlation between voltage changes and $[K^+]_o$ increase. We previously proposed that in our model the $[K^+]_o$ increase during EC seizures depends on the sustained firing of interneurons. $[K^+]_o$ rise could also be directly due to the activation of GABA type A receptors during massive GABA release (Bartolet and Morris 1992) by bursting interneurons. The $[K^+]_o$ changes may be responsible for restoring neuronal firing in principal neurons by promoting direct activation of AP firing. To verify whether AP firing could be directly evoked by increasing $[K^+]_o$, we locally applied a solution containing high potassium in the EC to obtain the effective concentration observed during seizures (Somjen and Giacchino 1985). As illustrated in Fig. 9, pressure ejection of a solution with 12 mM K$^+$ induced AP activity in principal neurons of superficial layers ($n = 10$). Compared with spontaneously generated control APs, the APs induced by the high-K$^+$ solution showed features similar to those described during the irregular firing phase.
phase of a bicuculline-induced seizure, indicating a possible common origin of the doublettes in similar conditions of K\textsuperscript{+}-induced depolarization. Spike doublettes and low-amplitude APs (arrows in Fig. 9) were often observed. As for APs observed during the irregular phase of the bicuculline-induced seizures, \(V_{\text{rest}}\) and \(V_{\text{thres}}\) of APs generated by local application of K\textsuperscript{+}/H11001 were depolarized, and \(V_{\text{max}}\) was less depolarized than control APs (Fig. 9C, left). Depolarizing and repolarizing slopes were also slowed in high K\textsuperscript{+}-induced spikes (Fig. 9C, right).

DISCUSSION

Based on the description of intra- and extracellular firing patterns, EC ictal events in our acute model of temporal lobe seizures feature three sequential and reproducible phases that are commonly observed during seizures in focal human epilepsies of the temporal lobe (Gnatkovsky et al. 2008; de Curtis and Gnatkovsky 2009). The fast activity phase at seizure onset is characterized by blockade of AP firing for several seconds and is coupled with fast activity sustained by enhanced inhibitory networks. During the irregular firing phase, reappearance of firing characterized by irregular APs of variable amplitude and frequency occurs. Rhythmic discharges characterized by burst firing that become both larger in amplitude and more synchronous with the progression of the seizure are the markers of the bursting phase. Irregular firing and rhythmic bursting are usually defined as “tonic” and “clonic” phases of a seizure. These clinically derived terms are not used in the present study because they define muscle activity patterns during motor seizures that are obviously not present during epileptiform discharges recorded in an in vitro preparation.

The concept that seizures can be generated by enhanced synchronization of inhibitory networks coupled with blockade of principal neuron firing is supported by findings obtained in different in vitro models of seizures (Cossart et al. 2001; Ziburkus et al. 2006; Derchansky et al. 2008; Gnatkovsky et al. 2008). Paired pulse tests previously performed in the EC showed that the percentage of inhibition, based on the reduction of the polysynaptic response, was about 30% at seizure onset (Gnatkovsky et al. 2008). Also, intracellular studies clearly showed a correlation between the appearance of fast activity (30 Hz) at seizure onset and the inhibition of principal cells of superficial layers that display a blockade of firing. Neurons in the deep layers of the medial EC did not show the same correlation between fast activity and IPSP generation.
Interneurons do fire at high frequency just during this initial phase, and their activity is temporally correlated to the appearance of fast activity and IPSP generation in principal cells of superficial layers (Gnatkovsky et al. 2008).

The present study describes the main features of APs in different neuronal populations and the changes in AP firing during the progressive seizure phases. We studied the mechanisms that restore and synchronize neuronal firing of principal EC neurons after paradoxical blockade observed at seizure onset. We tested the hypothesis that $[K^+]_o$ directly promotes regenerative AP firing in principal cells. Phase plot analysis of APs (Jenerick 1963; Bean 2007) was utilized for this purpose. This method implements a rapid evaluation of AP features by considering the first derivative of the AP vs. the absolute value of membrane voltage. The analysis of the changes of AP features during the transition from the fast activity phase to the irregular firing phase contributes to understand the dynamics of seizure development.

Within 2–10 s after interruption (fast activity phase), AP firing resumed in superficial and deep cells. Compared with the preictal period, a reduction of AP amplitude (reduction of $V_{\text{max}}$) was found primarily in superficial neurons. Following the pronounced depolarization of $V_{\text{rest}}$ during seizures, $V_{\text{thres}}$ also shifted to more depolarized values, and the time required for the AP to reach the maximum velocity slowed down. Small-amplitude AP with a depolarized $V_{\text{thres}}$ and slower-depolarizing slope are supposed to be generated in the dendrites of pyramidal neurons (Wong and Prince 1979; Benardo et al. 1982). Hot spots of regenerative inward channels were found in regions of the membrane remote from the somatic site (Johnston et al. 1996), where intracellular recording with sharp

**Fig. 6.** A: changes in AP features during a seizure simultaneously recorded from a neuron of the EC deep layers (bottom trace) and with an extracellular electrode (top trace). The 3 phases of the seizure are marked. The multipolar cell located in layer V is illustrated. Calibration bar, 250 $\mu$m. B: examples of APs expanded from the time points marked by the square boxes in A. The relative phase plots are illustrated on the right of each AP sample. APs before the seizure (a), during the irregular firing phase (b), and during the early and late bursting phase (c and d) are illustrated. Rmp = −71 mV.
electrodes is most likely performed. Dendritic APs are sustained by sodium conductances, since they were abolished by local application of the sodium channel blocker, tetrodotoxin (Wong and Prince 1979; Turner et al. 1991). The existence of regenerative APs sensitive to tetrodotoxin was confirmed by experiments in which dendrites and soma of pyramidal neurons were patched with two distinct electrodes. These studies performed on both hippocampal (Magee and Johnston 1995; Magee and Carruth 1999) and neocortical principal neurons (Stuart and Sakmann 1994) demonstrated that dendritic sodium APs show depolarized threshold, lower amplitude, and slight slowing of the slopes of depolarization and repolarization. It has been recently demonstrated (Sheffield et al. 2011) that nonsomatic persistent firing can be induced in different neuronal populations of rodent hippocampus after a period of high-frequency (30 Hz) stimulation. The spikelets generated after the conditioning period displayed the same characteristics of the doublette spikes that we observed in our preparation, with a more depolarized $V_{\text{thres}}$ and a two-component rising phase. Based on these findings, the observations of AP features of cells in the superficial layers of the EC are consistent with these data. Moreover, nonsomatic APs characterized by lower amplitude and depolarized threshold (compared with somatic APs) can be recorded in epileptogenic cortex in vivo (Pinault and Pumain 1985) and in vitro (Perrault and Avoli 1992; for review, see Pinault 1995).

Why should dendritic and not somatic APs be generated in our model during the transition from fast activity to the irregular firing phase? We demonstrated that at the onset of the seizure, principal neurons of the EC generate fast IPSPs at 25–30 Hz, possibly sustained by the robust feedforward inhibitory projection from the CA1-subicular region of the hippocampus (Gnatkovsky and de Curtis 2006; Gnatkovsky et al. 2008). Therefore, in the fast activity phase the soma of pyramidal and stellate neurons of superficial EC layers is hyperpolarized, and its excitability is dampened by the presence of IPSPs (Funahashi and Stewart 1998) that are presumably generated perisomatically (Witter and Wouterlood 2002). If tissue excitability is enhanced by the elevation of $[K^+]_o$ during the seizure (Traynelis and Dingledine 1988), APs could be generated in regions of the membrane (i.e., the proximal dendrites) that are not under the inhibitory clamp control of the mentioned IPSPs associated with fast activity. This hypothesis is also supported by the demonstration that possibly nonso-

Fig. 7. Averaged data of AP features during seizures obtained from 7 deep-layer neurons of the EC. The 4 graphs illustrate mean (± SE) values of $V_{\text{rest}}$, $V_{\text{thres}}$, $V_{\text{max}}$ and $V_{\text{repol}}$ before (preictal) and after (recovery) seizures and at 3 time points at the onset of the irregular firing phase and during the early and late bursting phase. Student’s $t$-test; *$P < 0.05$. 

\[ V_{\text{rest}} \]

\[ V_{\text{thres}} \]

\[ V_{\text{max}} \]

\[ V_{\text{repol}} \]
matic APs in our experiments were observed exclusively in superficial neurons and not in deep EC neurons that do not generate fast IPSPs during the fast activity phase (Gnatkovsky et al. 2008).

Thus, in our experimental conditions, regenerative APs could be generated ectopically in dendrites by the direct action of enhanced \([K^+]_o\). The changes in AP features observed during the irregular firing phase indeed paralleled the changes in \([K^+]_o\), suggesting a possible causal correlation between the two phenomena. High \([K^+]_o\) is a well-known epileptogenic factor (Jensen and Yaari 1988). Moreover, nonsomatic firing caused by direct depolarization of axonal or dendritic membrane via increases in local \([K^+]_o\) was demonstrated in hippocampal in vitro slices treated with 4-aminopyridine (Avoli et al. 1998). In our case the cell soma (V\text{rest} graph in Fig. 5) is depolarized and V\text{thres} is higher, whereas K\textsuperscript{+} channels are activated by the K\textsuperscript{+} application. In these conditions nonsomatic APs are generated.

Our experiments with local application of high K\textsuperscript{+} solution in close proximity to superficial neurons confirmed that the increase of \([K^+]_o\) in a restricted spot of EC is sufficient to generate APs with depolarized V\text{max}, lower V\text{max}, and slow depolarizing/repolarizing slopes similar to those observed during the seizures in the irregular firing phase. The decrease of V\text{max} and the slowing of repolarization during the irregular firing phase AP could be due to the influence of high \([K^+]_o\) on sodium and potassium channels. Increased \([K^+]_o\) reduces the driving force of outward K\textsuperscript{+} currents responsible for spike repolarization (such as the delayed rectifier and the A-current) and thus slows the outflow of K\textsuperscript{+}. Similarly, a reduction of driving force for the K\textsuperscript{+} modulates conductances that regulate resting membrane potential and promotes a depolarizing shift of V\text{rest}. Membrane depolarization is expected to inactivate sodium currents and reduce the driving force of moving sodium ions into the cells, giving K\textsuperscript{+} the depolarizing role held by sodium. These mechanisms slow down AP repolarization and reduce AP amplitude.

**Fig. 8.** A: correlation between firing pattern and changes in extracellular potassium concentration \([K^+]_o\) simultaneously recorded from EC superficial layers. The 3 phases of the seizure are reported at the bottom. B: changes in \([K^+]_o\) plotted on the changes in V\text{rest}, V\text{thres}, and V\text{max} (n = 5). Student’s t-test; *P < 0.05.
Studies on membrane AP generation in pyramidal neurons demonstrated the presence of a small hump component in the AP upstroke (called kink) interpreted as the reflection of the AP initiation in the initial segment of the axon (Colbert and Johnston 1996), where the higher density of sodium channels is present. In the APs recorded during our experiments in EC neurons, no kink was observed in control conditions, suggesting that APs in these neurons are generated at the soma and not in the axon. This could be due to the fact that the large majority of neurons in the superficial layers of the EC are not pyramidal neurons but have stellate or multipolar/multiform shape. Kink indeed was demonstrated in pyramidal neurons (Colbert and Johnston 1996). The revision of AP onset features of EC neurons based on the studies of Angel Alonso shows that multipolar and stellate EC cells have a more abrupt initiation of APs (Alonso and Klink 1993; Tahvildari and Alonso 2005), whereas EC pyramidal neurons show a slow prespike depolarization compatible with the kink associated with an AP originating from the axon initial segment.

During the irregular firing phase, spike doublettes were observed in 15 of 17 superficial neurons. The first AP of the spike doublettes (and the first AP of bursts during the bursting phase) showed depolarized $V_{\text{thres}}$, slower-depolarizing slope, and smaller amplitude. Compared with the first AP, the second AP of the doublette was generated at more depolarized membrane potential ($V_{\text{rest}}$) and showed a biphasic slope of the depolarizing phase associated with a further decrease of $V_{\text{thres}}$ and $V_{\text{max}}$. The very slow kinetic of depolarization and repolarization and the smaller amplitude suggest that these APs could be nonsodium spikes sustained by regenerative calcium conductances generated in the dendrites (Wong and Prince 1979; Llinás and Sugimori 1980). A typical feature of these calcium-dependent APs is the progressive voltage attenuation as they propagate from their site of generation to the soma (Schwindt and Crill 1997). A kink in the phase plot graphs was observed in the doublettes; as mentioned above, kink is not observed in control somatic APs before seizures, suggesting that large APs are generated not at the axon hillock but at the soma in EC neurons. Therefore, the kink observed in our experiments associated with small-amplitude APs assumes a different meaning. The double kink in the second AP of the doublettes suggests a mixed slow (possibly calcium-mediated) and fast (sodium-mediated) regenerative potential. The more distal slow AP could travel to more proximal dendritic portions where an “ectopic” sodium AP is generated. This hypothesis seems to be supported by the finding that on some occasions very low-amplitude APs with a higher $V_{\text{thres}}$ can be observed in isolation, as shown in Figs. 3Bc and 9A. Also, it has been reported that gap junction blockers such as mefloquine and carbenebolon can prevent the appearance of spikelets after the
induction of fast oscillatory activity in hippocampal interneurons (Schmitz et al. 2001; Sheffield et al. 2011).

Unlike superficial-layer neurons, deep-layer neurons show less AP changes during the transition to the irregular spiking phase, and they do not show spike doublettes. Even though the population of deep-layer neurons explored was small, no kink that showed all multipolar morphology was observed in these neurons. Even though a larger population of cells should ideally be considered to draw definitive conclusions, these data suggest that neither nonsomatic firing nor calcium spikes were generated in deep neurons. The observed difference between superficial and deep neurons could be due either to regional differences in $[K^+]_o$, or to differences in intrinsic regenerative AP properties. Preliminary experiments showed higher $[K^+]_o$ in superficial layers compared with deep layers. This could be due possibly to higher neuronal density and/or a larger number of (bursting) interneurons in superficial layers.

How principal neurons resynchronize during the transition from the irregular firing to bursting phase can be easily surmised. Propagation of distally generated APs to the somatic recording site could be facilitated by depolarization-dependent electrotonic diffusion. Moreover, reappearance of firing restores synaptic release of excitatory neurotransmitters from principal neurons that was transiently hindered by AP blockade during the fast activity phase. Recurrent synaptic and nonsynaptic excitatory interactions between principal cells (Jefferys 1995) enlarge progressive synchronization of EC neurons and promote bursting discharges. During the late bursting phase the first AP of the burst showed features similar to preseizure APs, promoting bursting discharges. During the late bursting phase the first AP of the burst showed features similar to preseizure APs, and slower APs were frequently observed after the first AP.

The experimental limitation of the in vitro isolated guinea pig brain preparation does not allow us to perform more detailed experiments on the cellular mechanisms of seizure generation/progression that are feasible in less complex preparations, such as brain slices. The close to in vivo condition of the isolated brain preparation interferes with the possibility to perform effective local application of blocking drugs because the whole limbic region of the isolated brain is massively activated during the seizure patterns that we describe. On the other hand, temporal lobe seizure patterns similar to those observed in the isolated guinea pig brain that mimic electrographic seizure features in humans are hardly reproducible in slices. Therefore, advantages and limitations of our preparation compared with others should be taken into consideration. In spite of these limitations, we can conclude that the APs that reestablish firing in superficial principal neurons are due to the direct effect of high $[K^+]_o$ on neuronal membrane, possibly at sites remote from the soma that is actively inhibited by fast IPSPs typically observed at this stage of the seizure. Firing emergence reactivates synaptic and nonsynaptic interactions between principal neurons and gradually drives irregular firing into bursting activity that extends across EC layers and possibly also to adjacent cortical limbic structures. Since similar sequences of events, characterized by fast activity followed by irregular spiking and synchronous bursting, are commonly observed in temporal lobe seizures in humans (see Engel 1993 and de Curtis and Gnatkovsky 2009 for review) and in chronic models of temporal lobe epilepsy (Bragin et al. 1999; Kharatishvili et al. 2006; Williams et al. 2009; Kadam et al. 2010), we propose that the cellular and network mechanisms described here could be relevant to elucidate the mechanisms of focal ictogenesis.

GRANTS
This study was supported by funding from the Italian Ministry of Health (Grant Giovani Ricercatori 2007-RF114) and by a grant from Telethon Italy (GGP07278).

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

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Backpropagation of action potentials generated at ectopic axonal


J Neurophysiol • VOL 106 • SEPTEMBER 2011 • www.jn.org

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