CHANGES IN ACTION POTENTIAL FEATURES DURING FOCAL SEIZURE DISCHARGES IN THE ENTORHINAL CORTEX OF THE IN VITRO ISOLATED GUINEA PIG BRAIN

Federica Trombin, Vadym Gnatkovsky and Marco de Curtis

Unit of Experimental Neurophysiology and Epileptology, Fondazione Istituto Neurologico Carlo Besta, via Celoria 11, 20133 Milano, Italy.

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Address for Correspondence

Marco de Curtis

Unit of Experimental Neurophysiology and Epileptology, Fondazione Istituto Neurologico Carlo Besta, via Celoria 11, 20133 Milano, Italy. Tel +39 02 23942280; e-mail decurtis@istituto-besta.it
ABSTRACT

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-minute arterial applications of the GABA<sub>A</sub> receptor antagonist, bicuculline. The onset of seizure is characterized by a paradoxical interruption of firing for several seconds in principal neurons, coupled with both enhanced inter-neuronal 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 here analyzed.

We utilized phase-plot analysis to characterize action potential features of entorhinal cortex neurons in different phases of a seizure. Compared to pre-ictal action potentials, resumed spikes in layer II-III neurons (n=17) during the early phase of the seizure-like discharge displayed i) depolarized threshold, ii) lower peak amplitude, iii) depolarized voltage of repolarization and iv) decelerated depolarizing phase, v) 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 non-somatic action potentials by increased extracellular potassium represents a crucial step to re-establish firing after an initial depression in an acute model of temporal lobe seizures. Resumed firing re-engages principal neurons into seizure discharge and promotes the transition towards the synchronized burst firing that characterizes the late phase of a seizure.
INTRODUCTION

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 pre-surgical studies aimed at defining the boundaries of the epileptogenic region in pharmaco-resistant 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 beta/gamma 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 post-ictal depression.

A similar progression of events were observed in chronic animal models of focal epilepsy (Bragin et al. 1999; Kharatishvili 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-minute arterial infusion of the GABAa receptor antagonist, bicuculline methiodide, promoted focal seizures in the hippocampal-parahippocampal 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. 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; Fujiwara-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 synergic effects produced by the [K⁺]ₒ elevation: i) a reduction of the efficacy
of GABAergic inhibition due to a depolarization of the GABAₐ-receptor mediated reversal potential
(Thompson and Gahwiler 1989) and ii) the induction of regenerative action potentials in principal cells.
The latter hypothesis was verified in the present study by performing phase-plot analysis of action
potential (AP) features in different populations of neurons during seizure progression.

Phase-plot analysis allows a simple evaluation of the membrane potential changes value (dV/dt)
versus voltage during AP generation (Jenerick 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 ictognesis in our experimental model.

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

METHODS

The method to isolate in vitro the guinea pig brain has been extensively described (de Curtis et al.
1991, 1998; Muhlethaler et al. 1993). Briefly, young adult Hartley guinea-pigs were anesthetized with
sodium thiopental (120 mg/kg, i.p.), the heart was exposed and the animal was perfused through the
ascending aorta with a 15°C saline solution (composition: NaCl 126 mM, KCl 2.3 mM, NaHCO₃ 26
mM, MgSO₄ 1.3 mM, CaCl₂ 2.4 mM, KH₂PO₄ 1.2 mM, glucose 15 mM and 3% dextran 70.000,
oxxygenated with a 5% CO₂/95% O₂ gas mixture; pH 7.1). The brain was carefully dissected out under
hypothermic conditions and was placed in an incubation/recording chamber. A polyethylene cannula (PE60) was inserted in the basilar artery and brain perfusion was restored through the resident arterial system with the above solution. The surgical procedures were performed at 15°C and the temperature was slowly (0.2°C/min) raised to 32°C to perform the experiment. Bicuculline methiodide (50 µM) was arterially perfused for 3 minutes to induce seizure activity (see Gnatkovsky et al. 2008). The experimental protocol was approved by the Ethical Committee on Animal Care. All efforts were done to reduce animal sufferance and the number of animal used.

Extracellular activity was recorded from the medial EC with glass pipettes filled with 0.9% NaCl. Intracellular recordings were performed from superficial layers (II-III) and from deep layers (V-VI) with sharp electrodes (input resistance 60-120 MΩ) filled with K-acetate 3M and 2% biocytine. Analog signals were digitalized with a 64-channel A/D board (National Instruments, TX) and were acquired and stored with the ELPHO® software. Off-line analysis was performed with specific LabView tools developed ad hoc by Dr. Vadym Gnatkovsky in our lab.

After the electrophysiological recording, cells were labeled with biocytine injected from the recording electrode. Brains were fixed in a 4% paraformaldehyde solution and were cut by vibratome. Slices (60 µm) were reacted with a standard protocol using a Staining kit for avidin-biotin complex (ABC-kit, Vector Laboratories, Burlingame, CA). The location of the cell in a specific cortical layer and its morphology were identified (Gnatkovsky et al. 2008).

The evaluation of [K+]o changes was performed with ion-sensitive electrodes. Double barreled glass capillaries with tips of 2-5 µm were filled with a K+ ionophore resin (Fluka 60031, Germany) and with NaCl 2M (reference capillary). The electrodes were calibrated before the experiment with different known [K+] solutions and the relative voltage increase was referred to a logarithmic increase in [K+] (see Librizzi et al. 2001). To measure potassium signals we used a high-input impedance differential amplifier (Biomedical Engineering, Thornwood, NY, US).

In order to reproduce atypical non-somatic AP firing, we performed local intra-EC injection of high 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⁺]₀ 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 1mm in EC superficial layers. Brief (100-300 ms) K⁺ puffs were delivered with a < 10µm tip pipette using a Picospritzer II (Parker Instrumentation, NJ). 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 (Jenerick 1963; Bean 2007). As illustrated in Figure 1, changes of the membrane potential with time (dV/dt measured as mV/ms - Y axis of the lower panel) are plotted against the instantaneous membrane potential (measured as mV - X axis). A single AP is represented as a loop in which the starting point represents the threshold membrane potential (Vthres), the extreme right peak is the maximal voltage amplitude (Vmax); the upper and lower parts of the loop describe the depolarization and repolarization phases (slopes), respectively. A software routine was developed in LabView by VG to rapidly obtain phase-plot graphs of either a single AP or groups of APs recorded during seizures. Phase-plot loops were smoothed with an Origin SP-line function. This analysis magnifies changes in Vthres, Vmax, repolarization potential (Vrepol) and resting membrane potential (Vrest) of APs recorded in different phases of a seizure.

Instantaneous AP count as a function of time was calculated by counting the number of APs over a 500-ms time window sliding by 60-ms step intervals on the intracellular traces (Figure 2). Averaged data are expressed as mean ± SE.

RESULTS

As previously shown, 3-minute perfusion of 50µM bicuculline methiodide induced seizure-like events in the medial EC of the isolated guinea pig brain (Uva et al. 2005; Gnatkovsky et al. 2008). A 35% decline in paired-pulse depression, but not a complete abolition of inhibition was found in the EC at
seizure onset (for details see Gnatkovsky et al. 2008). The typical ictal pattern observed with the extracellular recording electrode during this pharmacological manipulation consisted of three consecutive phases (Figure 2): at seizure onset, low voltage fast activity at 25-30Hz ($fa$ in Figure 2B) coupled to a slow negative potential; the second and third phases were respectively characterized by irregular firing and by rhythmic bursting 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 (Figure 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 IPSPs (bottom left panel in Figure 2B; 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 (bottom middle panel in Figure 2B). In the bursting phase, strong and coherent bursts of APs followed the extracellular activity (bottom right panel in Figure 2B), 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 Figure 5 and in Table 1A. Compared to pre-ictal spontaneous APs (Figures 3Ba and 4A), at the onset of the irregular firing phase APs diminished in amplitude ($V_{\text{max}}$ decreased), while $V_{\text{thres}}$ showed a depolarizing shift (Figures 3Bb and 4A). In this phase AP depolarizing and repolarizing phase was significantly slowed down ($p<0.001$ and $p<0.01$ respectively). The repolarizing potential ($V_{\text{repol}}$) was shifted toward depolarized values. In the following few seconds, spike doublettes appeared (Figure 3Bc and asterisks in 4B). 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 $V_{\text{max}}$. The second AP was evidently smaller in amplitude, started at
higher $V_{\text{thres}}$ and displayed a slower kinetic of repolarization (inner loops marked by arrows in Figures 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 Figure 4A and B). 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 (Figure 5). During the late bursting phase, $V_{\text{max}}$ and $V_{\text{thres}}$ of the first AP of each burst gradually returned to values similar to a pre-ictal AP and depolarizing/repolarizing slopes become faster compared to the irregular firing phase (Figure 3Bd and 5). A complete re-establishment of pre-ictal AP features was demonstrated in all neurons (Figures 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 Figure 4A. AP feature changes in a layer III neuron are illustrated in details in figure 4B.

Values of $V_{\text{rest}}$, $V_{\text{thres}}$, $V_{\text{max}}$, $V_{\text{repol}}$, and the slope of the depolarizing and the repolarizing phases that characterize AP features were quantified for the 17 superficial EC neurons and the data are reported in Figure 5 and Table 1A. Mean values calculated for the second APs of both AP doublettes and bursts are represented by white symbols. These population data demonstrate that, in comparison to the pre-ictal period, during the irregular firing and the initial bursting phases $V_{\text{rest}}$, $V_{\text{repol}}$ and $V_{\text{thres}}$ depolarized, $V_{\text{max}}$ hyperpolarized and depolarizing-repolarizing slopes decelerated. All these parameters recovered either during the late bursting phase or at the end of the seizure (left values in all plots of Figure 5). Larger differences were observed in the second APs of both spike doublette and burst. In particular, mean $V_{\text{thres}}$ 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 to 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 of the extra-somatic origin of the second spike, that 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)

**AP changes during seizure in neurons of deep EC layers.** AP features in 8 neurons from deep EC layers (V and VI) were also characterized. Figure 6A illustrates a seizure from a multipolar EC neuron of layer V (lower trace) simultaneously recorded with an extracellular electrode (upper trace).

The seizure developed with the typical three phases identified above. After seizure onset there was a brief interruption of firing that immediately recovered during the fast activity (fa) phase. We observed a slow depolarization in the \( V_{\text{rest}} \) during the irregular firing and bursting phase that was recovered during late bursting and after seizure end. The features of APs during the three typical phases (irregular firing, bursting and late bursting) were analyzed by phase-plot graphs and were compared to control pre-ictal and recovered APs (Figure 6B). Spike doublettes did appear in deep neurons neither during the *irregular firing phase* nor during any other seizure phase. Population analysis (Figure 7 and Table 1B) 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 were not substantially modified during seizures in deep layers 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 ± 0.32 mM to 7.7 ± 4.5 mM. The analysis of \([K^+]_o\) and \( V_{\text{rest}} \), \( V_{\text{thres}} \) and \( V_{\text{max}} \) values during seizures (Figure 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 Figure 9, pressure-ejection of a solution with 12 mM \(K^+\) induced AP activity in principal neurons of superficial layers \((n=10)\). Compared to spontaneously generated control APs, the APs induced by the high-\(K^+\) solution showed features similar to those described during the irregular firing phase of a bicuculline-induced seizure, indicating a possible common origin of the doublettes in similar condition of \(K^+\)-induced depolarization. Spike doublettes and low-amplitude APs (arrows in Figure 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 \(K^+\) were depolarized and \(V_{\text{max}}\) was less depolarized than control APs (left panel in Figure 9C). Depolarizing and repolarizing slopes were also slowed in high-\(K^+\) induced spikes (right panel in Figure 9C).

**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 neurons 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 (30Hz) 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 mEC 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 $\left[\text{K}^+\right]_o$ 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 versus the absolute value of membrane voltage. The analysis of the changes of APs 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 seconds after interruption (*fast activity phase*), AP firing resumed in superficial and deep cells. Compared to the pre-ictal 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, also $V_{\text{thres}}$ 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 electrodes are 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 non-somatic persistent firing can be induced in different neuronal population of rodent hippocampus after a period of high frequency (30Hz) stimulation. The spikelets generated after the conditioning period displayed the same characteristics of the doublette spikes 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, non-somatic APs characterized by lower amplitude and depolarized threshold (compared to 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 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 feed-forward 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 peri-somatically (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 to fast activity. This hypothesis is also supported by the demonstration that possibly non somatic 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, non-somatic 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 (\(V_{rest}\) graph in Fig.5) is depolarized and \(V_{thres}\) is higher while \(K^+\) channels are activated by the \(K^+\) application. In these conditions non somatic APs are generated.

Our experiments with local application of high-\(K^+\) 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 \(V_{thres}\), lower \(V_{max}\) and slow depolarizing/repolarizing slopes, similar to those observed during the seizures in the irregular firing phase. The decrease of \(V_{max}\) 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 \(K^+\) currents responsible for spike repolarization (such as the delayed rectifier and the A-current) and thus slows the outflow of \(K^+\).

Similarly, a reduction of driving force for the \(K^+\) modulates conductances that regulate resting membrane potential and promotes a depolarizing shift of \(V_{rest}\). Membrane depolarization is expected to inactivate sodium currents and reduce the driving force of moving sodium ions into the cells, giving \(K^+\) the depolarizing role held by sodium. These mechanisms slow down AP repolarization and reduce AP amplitude.
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), while EC pyramidal neurons show a slow pre-spike 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 out 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. When compared to 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 non-sodium spikes sustained by regenerative calcium conductances generated in the dendrites (Wong and Prince 1979; Llinás and Sugimori 1980). 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 not generated at the axon hillock, but at the soma in EC neurons. Therefore, the kink observed in our experiments associated to small amplitude APs assumes a different meaning. The double kink in the second AP of the doublettes suggest 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 in some occasion very low amplitude APs with a higher $V_{\text{thres}}$ can be observed in
isolation, as shown in Figures 3Bc and 9A. Also it has been reported that gap junction blockers such
as mefloquine and carbenoxolone 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 layers 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
depth-layer neurons explored was small, no kink was observed in these neurons that showed all
multipolar morphology. Even though a larger population of cells should be ideally considered to draw
definitive conclusions, these data suggest that neither non-somatic 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 $[\mathcal{K}'_\mathcal{O}]$ or to differences in intrinsic regenerative AP properties.
Preliminary experiments showed higher $[\mathcal{K}'_\mathcal{O}]$ in superficial layers compared to deep layers. This could
be possibly due to higher neuronal density and/or larger number of (bursting) interneurons in
superficial layers.

How principal neurons re-synchronize during the transition from 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 non-synaptic
excitatory interactions between principal cells (Jefferys 1995) enlarge progressively synchronization of
EC neurons and promote bursting discharges. During the late bursting phase, the first AP of the burst
showed features similar to pre-seizure 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 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 massive 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 in comparison with others should be taken in consideration. In spite of these limitations, we can conclude that the AP that re-establish 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 re-activates synaptic and non-synaptic 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 (Engel 1993; 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 here described could be relevant to elucidate the mechanisms of focal ictogenesis.

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Table 1

Average values of resting membrane potential ($V_{\text{rest}}$), threshold membrane potential ($V_{\text{thres}}$), maximal voltage peak of the AP ($V_{\text{max}}$); repolarization potential ($V_{\text{repol}}$), depolarizing and repolarizing AP slopes (see Figure 1) of principal neurons of the superficial EC layers (A; n=17) and deep layers (B; n=7). Data measured with references to AP recorded before the onset of arterial perfusion of bicuculline are expressed as mean ± SE.

**A. Neurons of superficial EC layers**

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<th>Recovery</th>
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<td>$V_{\text{rest}}$ (mV)</td>
<td>6,7±1,1</td>
<td>18,6±2,6 **</td>
<td>20,2±3,1</td>
<td>20,6±2,8 ***</td>
<td>14,9±1,6 ** 5,1±0,7</td>
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<tr>
<td>$V_{\text{thres}}$ (mV)</td>
<td>19,7±2,7</td>
<td>21,5±1,7</td>
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<td>21,8±1,8 23,8±2,1</td>
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<td>$V_{\text{max}}$ (mV)</td>
<td>69,0±2,9</td>
<td>62,2±2,1</td>
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<td>63,8±1,9</td>
<td>70,9±2,5 71,7±3,2</td>
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<td>depolarizing slope (mV/ms)</td>
<td>20,2±1,5</td>
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<td>10,0±0,8 ***</td>
<td>12,9±1,4 *** 18±1,9</td>
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<td>$V_{\text{repol}}$ (mV)</td>
<td>22,1±2,6</td>
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<td>-6,4±0,6</td>
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<td>-3,3±0,3 ***</td>
<td>-4,4±0,6 ** -5,4±0,3</td>
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**B. Neurons of deep EC layers**

<table>
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<tr>
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<td>$V_{\text{thres}}$ (mV)</td>
<td>12,6±2,6</td>
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<td>depolarizing slope (mV/ms)</td>
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<td>$V_{\text{repol}}$ (mV)</td>
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</table>

* p < 0,05 ** p < 0,01 *** p < 0,001
Table 2.

Synoptic table of cells recorded in superficial and deep layers of the medial EC. The depth of cellular soma was calculated from the surface. Cell morphology was evaluated by histological reconstruction after intracellular biocytine injection.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Depth of cell (μm)</th>
<th>Cell morphology</th>
<th>Number of cells</th>
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<tr>
<td>II</td>
<td>200-350</td>
<td>Pyramidal-like</td>
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<td></td>
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<td>Stellate</td>
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<td>350-500</td>
<td>Pyramidal-like</td>
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<tr>
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<td>Multipolar</td>
<td>8</td>
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</table>
FIGURE LEGENDS

Figure 1. Phase plot analysis of action potentials (APs). In the upper panel an AP is illustrated. In the lower panel a phase plot is shown. $V_{\text{rest}}$ = resting membrane potential; $V_{\text{thres}}$ = threshold membrane potential; $V_{\text{max}}$ = maximal voltage peak of the AP; $V_{\text{repol}}$ = repolarization potential. The upper and the lower parts of the loop describe the depolarization and repolarization phases, respectively.

Figure 2. AP firing during a seizure induced in the entorhinal cortex (EC) of the isolated guinea pig brain by a 3-minute perfusion of 50 µM bicuculline. Extracellular (upper trace in panel A) and intracellular (lower trace in panel A) activity recorded in a pyramidal-like type neuron of the EC superficial layers. The arrow marks the time of onset of the seizure. The dotted line marks the resting membrane potential, ($rmp$ -64.9 ± 1.451 mV). In panel B instantaneous AP frequency during seizure progression is shown. The seizure is subdivided in three 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 fast activity phase. In panel 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 m-EC is shown on the right at 20X and 40X magnifications. Calibration bar 250µm.

Figure 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 m-EC 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 doublettes (c), during the bursting phase (d) and 20 minutes after the seizure (e) are illustrated. Rmp = -62 mV.

Figure 4. Changes in AP features during a seizure recorded in superficial EC layers, illustrated by phase-plot analysis. A. Loop graphs of AP recorded before the seizure (thin continuous line), during spike doublettes (thick continuous line) and after the end of the seizure (dotted lines) in a principal EC neurons of superficial layers. The first and the second AP within a doublette are identified. The double component of the depolarizing slope is marked by the arrows. B. AP features during the transition from the fast activity phase to the irregular firing phase is illustrated for a multipolar neuron of layer II (rmp = -71 mV). Superimposed phase-plots of the identified APs are illustrated in the lower part of the figure.

Figure 5. Averaged data of AP features during seizures obtained from 17 superficial layer neurons of the EC. The six panels illustrate mean (±SE) values of the resting membrane potential (Vrest), threshold potential of (Vthres) and maximal amplitude of APs (Vmax) and repolarizing potential (Vrepol), depolarizing and repolarizing slopes, before (preictal) and after (recovery) seizures and at three time points at the onset of the irregular firing phase and early and late during the bursting phase. White symbols mark the mean values measured during the second AP of either a spike doublette (irregular firing) or an early bust (bursting onset) recorded during the transition between the irregular firing phase and the bursting phase. T-student test p< 0.05 (*), p< 0.01 (**), p< 0.001 (***)

Figure 6. A. Changes in AP features during a seizure simultaneously recorded from a neuron of the EC deep layers (lower trace) and with an extracellular electrode (upper trace). The 3 phases of the seizure are marked. The multipolar cell located in layer V is illustrated. Calibration bar 250µm. 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), during the early and late bursting phase (c and d) are illustrated. Rmp = -71 mV.

Figure 7. Averaged data of AP features during seizures obtained from 7 deep layer neurons of the EC. The four panels illustrate mean (±SE) values of Vrest, Vthres, Vmax and Vrepol before (preictal) and after (recovery) seizures and at three time points at the onset of the irregular firing phase and during the early and late bursting phase. T-test p< 0.05 (*), p< 0.01 (**), p< 0.001 (***).

Figure 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 in the bottom. B. Changes in [K+]o plotted on the changes in Vrest, Vthres, Vmax (n=5). T-test p< 0.05 (*), p< 0.01 (**), p< 0.001 (***).

Figure 9. A. AP features of EC superficial neuron evoked by local pressure ejection of a bolus of high-K+ solution (12 mM) in the EC, close to the intracellular recording site (see schematic drawing). In a and b, spontaneous AP recorded before the application of the high-K+ solution and AP firing induced by the high-K+ ejection (grey bar) are shown, respectively. B. Phase-plot of control APs (thin lines) and APs induced by the high-K+ (thick line) are shown in the panel on the left. A spike doublette and low-amplitude APs are marked by the arrows. C. Quantification of the differences in Vrest, Vthres, Vmax and Vrepol (left panel) and depolarizing/repolarizing slopes (right panel) measured in spontaneous APs (black columns) and in APs induced by local EC ejection of high-K+ solution (white columns; n=10).
A

![Diagram of neural activity](image)

B

- **a)** pre-ictal spikes
- **b)** irregular firing
- **c)** doublettes
- **d)** bursting
- **e)** recovery

![Graphs of membrane potential change](image)
A

rmp

[\text{seizure onset}]

faster
irregular firing
bursting

B

$V_{rest}$

$V_{hres}$

$V_{max}$

$[K^+]_o$

8 mM

6

4
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