Voltage Imaging of Epileptiform Activity in Slices From Rat Piriform Cortex: Onset and Propagation

REZAN DEMIR, LEWIS B. HABERLY, AND MEYER B. JACKSON

Department of Physiology and Center for Neuroscience, University of Wisconsin Medical School, Madison Wisconsin 53706

Demir, Rezan, Lewis B. Haberly, and Meyer B. Jackson. Voltage imaging of epileptiform activity in slices from rat piriform cortex: onset and propagation. J. Neurophysiol. 80: 2727 ± 2742, 1998. The piriform cortex is a temporal lobe structure with a very high seizure susceptibility. To investigate the spatiotemporal characteristics of epileptiform activity, slices of piriform cortex were examined by imaging electrical activity with a voltage-sensitive fluorescent dye. Discharge activity was studied for different sites of stimulation and different planes of slicing along the anterior-posterior axis. Epileptiform behavior was elicited either by disinhibition with a γ-aminobutyric acid-A receptor antagonist or by induction with a transient period of spontaneous bursting in low-chloride medium. Control activity recorded with fluorescent dye had the same pharmacological and temporal characteristics as control activity reported previously with microelectrodes. Simultaneous optical and extracellular microelectrode recordings of epileptiform discharges showed the same duration, latency, and all-or-none character as described previously with microelectrodes. Under all conditions examined, threshold electrical stimulation applied throughout the piriform cortex evoked all-or-none epileptiform discharges originating in a site that included the endopiriform nucleus, a previously identified site of discharge onset. In induced slices, but not disinhibited slices, the site of onset also included layer VI of the adjoining agranular insular cortex and perirhinal cortex, in slices from anterior and posterior piriform cortex, respectively. These locations had not been identified previously as sites of discharge onset. Thus like the endopiriform nucleus, the deep agranular insular cortex and perirhinal cortex have a very low seizure threshold. Additional subtle differences were noted between the induced and disinhibited models of epileptogenesis. Velocity was determined for discharges after onset, as they propagated outward to the overlying piriform cortex. Propagation in other directions was examined as well. In most cases, velocities were below that for action potential conduction, suggesting that recurrent excitation and/or ephaptic interactions play a role in discharge propagation. Future investigations of the cellular and organizational properties of regions identified in this study should help clarify the neurobiological basis of high seizure susceptibility.

INTRODUCTION

Brain regions with unusually low seizure thresholds provide unique opportunities for the study of epileptiform activity. Brain slices prepared from these regions have been used as in vitro models to investigate the cellular correlates of epileptiform discharges and to test the influence of environmental variables (Schwartzkroin 1986; Traub and Miles 1991). Moreover, studies of the physiology and anatomy of highly excitable brain regions offer the hope of understanding what properties of a nervous system are responsible for a high susceptibility to epilepsy. This approach depends on precise knowledge of the spatiotemporal pattern of discharge onset and spread, to pinpoint specific structures with different roles in seizure generation. For this reason, a number of laboratories have used imaging techniques in conjunction with voltage-sensitive dyes to investigate epileptiform activity (Albowitz et al. 1990; Colom and Saggau 1994; London et al. 1989; Sutor et al. 1994).

The piriform cortex (PC) is an example of a brain region with an unusually strong tendency to generate seizures in vivo and epileptiform discharges in vitro (Hoffman and Haberly 1991, 1993, 1996; McIntyre and Wong 1986; Piredda and Gale 1985; Stevens et al. 1988). Epileptiform discharges generally appear in deep structures before spreading to the overlying superficial layers of the PC (Hoffman and Haberly 1991). The extensive associational projections from the PC to areas involved in temporal lobe epilepsy, including amygdaloid cortex, entorhinal cortex, and adjacent neocortex (Haberly and Price 1978; Luskin and Price 1983) provide an important putative pathway for the generalization of seizures (Halonen et al. 1994; Racine et al. 1988).

Structures in the deep piriform region, including the endopiriform nucleus (En), deep layer III, and claustrum, have been identified as regions with especially low thresholds for the generation of epileptiform discharges (Hoffman and Haberly 1991, 1996). Microelectrode mapping showed that the dorsal edge of the endopiriform nucleus has the shortest latency for discharge onset and is therefore the site where discharge initiation occurs (Hoffman and Haberly 1993). Voltage imaging techniques, which already have been used to study other aspects of circuitry in the PC of a variety of species (Cinelli and Kauer 1992; Litaudon and Cattarelli 1996; Sugitani et al. 1994), can provide a direct view of the onset and spread of epileptiform discharges. In the present study, we have used imaging technology to investigate interictal-like activity in slices of the rat PC. We used this technique to map the site of onset and determine its uniqueness under a variety of experimental conditions, including stimulus site and region from which slices are prepared. Epileptiform activity was initiated by two different protocols, which are referred to here as disinhibition and induction. In the disinhibition protocol, the γ-aminobutyric acid-A (GABA_A) receptor antagonist bicuculline methiodide is added to the saline perfusing slices while recordings are...
underway. In the induction protocol, slices are subjected to a transient period of burst activity in low-chloride saline and then returned to normal saline for recording (Hoffman and Haberly 1989). Induction results in epileptiform activity that strongly resembles that seen in kindled epileptogenesis. For all of the conditions examined here, the site of onset of epileptiform discharges evoked by threshold electrical stimulation included the En. However, the precise location within the En varied, and in the induction model, discharges appeared simultaneously in a newly identified site of onset in neighboring neocortex as well as in the En. In anterior piriform cortex slices, this new site was adjacent to the En in layer VI of the deep agranular insular cortex (AI). In posterior slices, this new site was adjacent to the En in layer VI of the anterior perirhinal cortex (PRh). Preliminary accounts of this work have been presented (Demir et al. 1994, 1996).

METHOIDS

Slice preparation

Slices of PC were prepared as described previously (Hoffman and Haberly 1989, 1991). Male Sprague-Dawley rats weighing 175–220 g were decapitated under CO2-induced narcosis. A block of brain from the right hemisphere containing piriform cortex was cut in a plane that was shifted slightly from coronal to pass perpendicular to the brain surface. This block then was sectioned with a Vibratome at a thickness of 350 μm, while immersing in chilled artificial cerebrospinal fluid (ACSF) consisting of (in mM) 124 NaCl, 5 KCl, 1.2 KH2PO4, 2.4 CaCl2, 1.3 MgSO4, and 10 glucose bubbled with 95% O2-5% CO2 (carbogen). Before recording, slices were kept at room temperature in vials containing carbogen-bubbled ACSF.

Diagrams of anterior, intermediate, and posterior PC slices are shown in Fig. 1 to point out the principal structures that will be referred to in this study. Slices were obtained from stereotaxic levels −0.70 to −1.60 mm (anterior slices), −1.80 to −0.70 mm (intermediate slices), and −2.80 to −1.80 mm (posterior slices). In anterior slices, defined by the presence of the lateral olfactory tract (LOT), the AI and subjacent claustrum adjoin the PC and En laterally (Fig. 1A). In posterior slices, the PRh, which corresponds to the posterior AI of Krettek and Price (1977), forms the lateral border of PC and En (Fig. 1C). The term PRh will be used to distinguish that part of the perirhinal cortex lying adjacent to piriform cortex (Paxinos and Watson 1986) from the posterior portion of perirhinal cortex lying adjacent to entorhinal cortex. Intermediate slices are at the level of transition between the AI and PRh, where cytoarchitecture is intermediate in form and the claustrum is small or absent (Fig. 1B).

During recordings slices were perfused with carbogen-bubbled ACSF and maintained at a temperature of 34°C. Slices were stimulated with 200-μs current pulses delivered by a monopolar constant stimulus isolator through ACSF-filled glass micropipettes with tip diameters of ~50 μm.

Generation of epileptiform behavior

Two protocols were used to make slices produce epileptiform discharges. 1) Induction. Slices were induced by treatment in a low-CI− ACSF (in which 93% of the CI− was substituted by isethionate) for 30–70 min at 34°C. This treatment causes spontaneous bursting by reversing CI−-mediated inhibitory synaptic potentials. After returning to control ACSF (with normal [Cl−]) epileptiform bursts could be evoked in a manner similar to that seen after induction by bursting in zero-Mg2+ solutions (Hoffman and Haberly 1989, 1991). Induction has been employed as an in vitro model for kindled epileptogenesis. 2) Disinhibition. GABA receptors were blocked by bath application of 5–10 μM bicuculline methiodide. It should be noted that induction and disinhibition are quite different in terms of protocol as well as mechanism. The induction model is a treatment before recording and is based on an N-methyl-D-aspartate receptor-mediated form of long-term plas-
ticity of synaptic circuitry (Hoffman and Haberly 1989). The disinhibition model is a drug treatment during recording and is based on direct blockade of rapidly activated inhibitory synaptic receptors. In general, the epileptiform discharges we observed in these models resembled previously described epileptiform activity in piriform cortex slices under these or similar conditions. Lowering [K+] and [Ca2+] to more physiological levels has been shown previously to leave epileptiform activity in this preparation qualitatively unchanged (Hoffman and Haberly 1991).

Careful variation of the stimulus current was used to determine the threshold for epileptiform discharge generation. Once the threshold was precisely determined, we could evoke sub- and suprathreshold epileptiform responses with roughly equal probability using the same stimulus current. Unless otherwise noted, discharges were evoked with currents at or slightly above threshold.

**Drug application**

Drugs were applied by addition to the ACSF superfusing the preparation. 6,7-Dinitroquinoxaline-2,3-dione (DNQX) and D,L-2-amino-5-phosphonovaleric acid (APV) were obtained from the Research Biochemical (Natick, MA), and bicuculline methiodide was obtained from Sigma (St. Louis, MO).

**Imaging of voltage**

Voltage was imaged with the aid of the voltage-sensitive fluorescent dye RH414 (4-4’,5-diethylaminophenylbuta-1’,3’,4’-dienyl-γ-triethylammonium-propyl pyridinium bromide; Molecular Probes, Eugene, OR). Slices were stained by treating with 200 µM RH414 in carbogen-bubbled ACSF for 30–45 min.

The photodiode system used for these studies follows the design of Wu and Cohen (1993) and has been described in detail by Jackson and Scharfman (1996). A schematic of this system is shown in Fig. 2. An upright Reichert Jung Diastar epifluorescent microscope (Leica, Deerfield, IL) was used with illumination from a 100-W tungsten-halogen lamp. Light from this source passed through an excitation filter (475- to 565-nm band-pass) and was reflected off a 570-nm dichroic mirror. A microscope objective focused the light onto the preparation and collected fluorescent light. The fluorescent light then was transmitted first through the dichroic mirror and then through a 610-nm long-pass emission filter, after which it was imaged in a hexagonally arranged photodiode-fiber optic camera (Chien and Pine 1991) with 464 photodiodes. The output of each detector was amplified individually, digitized, and stored in a Pentium computer. Photodiode currents were amplified to a final level of 0.2 V/pA of photocurrent, high-pass filtered with a 100- or 500-ms time constant, and low-pass filtered with a corner frequency of 300 or 500 Hz. Optical signals were presented as percentage change in fluorescence normalized to resting light intensity and were on the order of 0.1–0.3%.

A Zeiss Fluar ×5 objective (N. A. = 0.25) was used for fluorescence recording. With this objective the distance between centers of the fields imaged by neighboring photodetectors was 144 µm. A Zeiss Fluar ×2.5 objective (N. A. = 0.075) was used to take standard trans-illuminated video pictures of slices. A movable mirror was inserted in front of the photodiode detector to reflect a transmitted light image to a CCD camera (Fig. 2). This image was then read into the computer with a frame-grabber (Data Translation, Marlboro, MA).

**Data acquisition and analysis**

Voltage-sensitive dye fluorescence was digitized and analyzed with the computer program NeuroPlex (OptImaging, Fairfield, CT) that runs under IDL (Research Systems, Boulder, CO). Optical signals were acquired at a sampling rate of 0.944 ms per frame of

**FIG. 2.** Instrumental setup for imaging voltage is shown schematically. Light from a 100-W bulb passes through the 475- to 565-band-pass excitation filter and is reflected into the back of the objective and onto the preparation by the 570-nm dichroic mirror. Fluorescent light collected by the objective passes through the dichroic mirror and through a 610-nm long-pass emission filter. Fluorescent light is focused either onto the 464-element photodiode-fiber optic camera or the CCD camera, depending on the position of the movable mirror. Both the fluorescence signals and the CCD image are read into a computer.

464 detectors with a DAP 3200e/214 A/D converter (Microstar, Bellevue, WA). After data acquisition, optical signals were superimposed on CCD video images using an overlay program written in IDL. Test patterns of pinholes were collected with the CCD camera and the photodiode system before each experiment to determine the proper alignment of the two images. The overlay procedure aligned the images to within approximately one-half the distance between neighboring detectors.

The site of onset of epileptiform discharges was determined in imaging records by two different methods. In a slower manual method, individual fluorescence traces from each photodiode were examined visually to determine the time at which the discharge was first visible above baseline noise. The first traces to show the discharge then could be marked by hand in an overlay of traces on a video image such as shown in Figs. 5 and 9. A faster automated method also was used in which a computer program drew a contour around the first detectors at which the fluorescence signal crossed a threshold of 50–70% of the maximal discharge amplitude. The contour then was overlaid on video images with a program written in IDL. One can envision one of these contours in the color-coded intensity maps in Fig. 9B. A site of onset contour would surround the green-yellow region in frame 24. Sites of onset obtained either by manual or automated analysis were similar.

**Extracellular recordings**

Extracellular field potentials were recorded simultaneously with fluorescence using glass microelectrodes filled with 1 M KCl (re-
FIG. 3. Voltage-induced changes in dye fluorescence are shown from selected locations of an anterior slice under control conditions. A: video image shows the locations from which fluorescence traces were taken, as well as the site in layer Ib where a 200-μA, 200-μs stimulus was applied (indicated by arrow). Arrows show precise locations. Deep and superficial levels of layer III are denoted as d. III and s. III, respectively. Other labels are as in Fig. 1. Each trace represents the fluorescence recorded in a single trial by a single detector.

C: single detector trace from layer Ib of a different control slice is shown before and after application of 100 μM D,L-2-amino-5-phosphonovaleric acid (APV) and 50 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX). A 400-μA, 200-μs stimulus was applied in the LOT, and 5 trials were averaged to produce the traces shown. These excitatory amino acid antagonists blocked the stimulus-evoked fluorescence changes, demonstrating that they are mediated by excitatory amino acid receptors.

RESULTS

Spatial distribution of optical signals

Fluorescence signals revealed the spread of change in membrane potential in a PC slice after electrical stimulation (Fig. 3). A video picture of the slice is shown in Fig. 3A together with fluorescence traces from different locations in Fig. 3B. Under control conditions stimulation in layer Ib-evoked fluorescence changes that were largest near the stimulus pipette (site marked as arrow) and declined with distance from the site of stimulus. These control responses are coupled tightly to the electrical stimulus. They started within 1 or 2 ms of stimulation, peaked within 10 ms, and returned to baseline within 75–100 ms. The responses were graded with stimulus intensity (data not shown), as previously observed with this recording system in hippocampal slices (Jackson and Scharfman 1996). Thus under control conditions, stimulus-evoked fluorescence changes have no epileptiform component. Responses were blocked by the excitatory amino acid antagonists DNQX and APV (Fig. 3C), indicating that the depolarizations depend on the activation of excitatory amino acid receptors. Furthermore, when recordings were made with a high-pass time constant of 500 ms, it was possible to see a slow hyperpolarizing potential in layers Ia
ONSET OF EpILEPTIFORM DISCHARGES IN PIRIFORM CORTEX 2731

... that local inward currents underlying the negative-going field potential are responsible for the membrane depolarization signaled by the fluorescence change. The biphasic nature of the field potential indicates that cell membranes near the electrode pass inward current at the onset of the epileptiform burst and outward current after the burst is underway.

The distribution of optical responses through a slice can be viewed by superimposing fluorescence traces on a video image (Fig. 5). The optical traces in this figure are laid out according to the hexagonal arrangement of detectors used to record fluorescence. The overlay in Fig. 5A shows the decrement in response in a control slice as the distance from the site of stimulation increases. This information is similar to that in Fig. 3, A and B, but with more spatial detail. The extent of spread is variable depending on stimulus strength, but in general, stimulus-induced fluorescence changes can be seen at distances of up to ~1 mm from the stimulating electrode.

The spread of fluorescence signals is very different in slices showing epileptiform activity, where responses can be seen over much greater distances. Figure 5B shows responses in a slice disinhibited with 5 μM bicuculline methiodide. Selected traces under epileptiform conditions are shown in Figs. 6–8 to illustrate the features of this activity, but Fig. 5 helps visualize the spread of the epileptiform discharge directly in a slice of PC. It is notable that fluorescence changes are especially large in deeper layers, where the susceptibility for epileptogenesis is high (Hoffman and Haberly 1991, 1993, 1996; Stevens et al. 1988). These larger...
Threshold behavior of epileptiform discharges. *A*: As the stimulus current is increased from 10 to 60 μA (currents to the right of En traces) the local response in layer II increases in a graded fashion. Local response is barely visible in En. Threshold for epileptiform discharge in this experiment was 60 μA (for 200-μs duration), and the discharge is shown with fluorescence traces from both layer II and En. *B*: further increases in stimulus current above threshold reduced the latency of epileptiform discharges. These recordings were from an induced intermediate slice stimulated in layer III. Traces are single trials with signals from 3 (layer II) and 4 (En) neighboring detectors averaged.

responses in Fig. 5B were triggered by a weaker stimulus current (150 μA) than that used in the control experiment of Fig. 5A (300 μA). Similar results also were obtained with epileptiform discharges in the induced model (data presented below).

### All-or-none nature of epileptiform discharges

Stimulus current was varied to demonstrate the all-or-none character of epileptiform discharges. Figure 6A shows fluorescence signals from the En and layer II of an induced slice. Traces from these two sites are displayed for a series of stimuli ranging from 10 to 60 μA to illustrate the responses to sub- and suprathreshold stimulation in the two locations. With stimulus currents below the threshold for epileptiform activity, local responses were seen mainly in the vicinity of the stimulus electrode in layer III. These subthreshold responses were graded and were similar to the responses in control slices (Fig. 3). With stimulus currents greater than or equal to the threshold for epileptiform activity (60 μA in the experiment Fig. 6A), the initial responses were followed by an all-or-none epileptiform burst lasting ~200 ms. The amplitudes of the fluorescence changes associated with epileptiform discharges were largest in the En and deep neocortical layers. These amplitudes were independent of stimulus current once the threshold was exceeded (Fig. 6B). For stimulus currents at or slightly above threshold, the epileptiform burst had a latency of ~150 ms, but as the stimulus current was increased, the latency became shorter. These data show that epileptiform discharges recorded with voltage-sensitive fluorescent dye have the same properties as epileptiform excitatory postsynaptic potentials (Hoffman and Haberly 1989, 1991).

### Discharge onset and propagation

Fluorescence traces from En, overlying PC, and deep neighboring neocortex show clearly that the epileptiform discharge appeared first in the En (Fig. 7). These traces were taken from intermediate slices, where the site of discharge onset does not include adjoining neocortex (see following text). These data illustrate the point made earlier (Fig. 5) that fluorescence changes associated with discharges have larger amplitudes in deep layers as compared with superficial layers. The duration of the discharge in the induced slice was less than in the disinhibited slice, reflecting a consistent difference between these two in vitro models of epilepsy (see also Fig. 12). This result implies different mechanisms of discharge termination in induced and disinhibited
slices. In particular, the shorter duration of epileptiform bursts in induced slices implies a role for termination by GABA<sub>A</sub> receptors. In contrast, in disinhibited slices where GABA<sub>A</sub> receptors are blocked, other processes must terminate epileptiform bursts, and these processes are presumably slower.

The propagation of epileptiform bursts is illustrated with a series of fluorescence traces along the deep-to-superficial axis in a disinhibited slice (Fig. 8B). This experiment was carried out in the same slice used to obtain the control responses shown in Fig. 3. The fluorescence traces showing the subthreshold response in Fig. 8B reveal the decrease in local response amplitude with distance from the site of stimulus (layer Ib), and this decrease resembles that shown in the same slice under control conditions (Fig. 3). With a suprathreshold stimulus there was still a local response, and the same slice under control conditions (Fig. 3). With a suprathreshold stimulus there was still a local response, and the same slice under control conditions (Fig. 3).

FIG. 7. Temporal relationships among voltage changes in En, deep AI/PRh<sub>a</sub>, and layer III in 2 intermediate slices. Fluorescence traces were taken from 3 locations in induced (A) and disinhibited (B) slices to show that the epileptiform discharge appears in the En (b) before it appears in layer III (a), and AI/PRh<sub>a</sub> (c). A 100-µA stimulus was applied in layer III (A) and a 29-µA stimulus was applied in layer Ib (B). Traces shown here are averages of a hexagonal arrangement of 7 neighboring detectors in both experiments. Note that the different delays between the epileptiform discharges in traces (b) and (a) of ~30–40 ms in A and ~20 ms in B reflect optical responses taken from sites at different distances from the site of onset in the 2 experiments.

Mapping discharge onset in anterior, intermediate, and posterior slices

To display the site of onset of the epileptiform burst more precisely, we focused on the first time point at which the epileptiform event was evident (e.g., frame 24 of Fig. 9B) and drew a contour around the region where the fluorescence amplitude was >50–70% of the maximum reached at that location (see METHODS). This contour then was superimposed on a video picture of the slice to show the precise location at which the epileptiform event originated. A Nissl-stained picture of the same slice then was used to aid in the identification of different anatomic structures.

In induced anterior and posterior slices, the site of onset included the dorsal part of the En, as well as part of the adjacent neocortex. In anterior slices (n = 8), the site of onset extended into the adjacent parts of layer VI of the AI (Fig. 10A), and in posterior slices (n = 18) the site of onset extended into layer VI of PRh<sub>a</sub> (Fig. 11A). Discharge onset in the deep AI and PRh<sub>a</sub> is a new finding that suggests that in induced slices epileptiform discharges are initiated simultaneously in a region that includes two contiguous but cytoarchitecturally distinct sites.

In disinhibited anterior (n = 6) and posterior (n = 5) slices the epileptiform discharge began within the En with little if any tendency for onset at the dorsal edge (Figs. 10B and 11B). In neither anterior nor posterior disinhibited slices did the site of onset include adjacent neocortex. In neither induced nor disinhibited anterior or intermediate slices were epileptiform bursts seen to originate in the claustrum.

These points concerning site of onset can be seen by comparing the timing of individual fluorescence traces from the relevant regions in two anterior slices (Fig. 12). In an induced slice in Fig. 12A, superimposing a trace from the dorsal edge of the En with traces from the deep AI and claustrum shows that the epileptiform discharge appeared...
FIG. 8. Spread of epileptiform discharge. A: video image of an anterior slice shows different locations from which fluorescence traces were taken. Slice is the same as in Fig. 3. B: fluorescence traces from these locations are superimposed for sub- and suprathreshold responses to an 80-μA stimulus applied to layer Ib (site indicated by ⬤). Slice was disinhibited with 5 μM bicuculline methiodide. Traces are single trial responses recorded by single detectors.

Simultaneously in the En and deep AI, however, in a disinhibited slice the fluorescence clearly rose in the En before the deep AI (Fig. 12B). The signals in the claustrum lagged behind the signals from En and AI in both induced and disinhibited anterior slices.

Traces within the site of onset showed a ramplike depolarization from baseline before the onset of the epileptiform discharge, regardless of whether the onset occurred in the En or the deep AI (Fig. 12). This ramplike buildup corresponds well with the slow onset of epileptiform discharges in the En, where spike activity within a network of cells connected by excitatory synapses gradually increases prior to the discharge (Hoffman and Haberly 1993). Figure 12 also illustrates the point made earlier that epileptiform discharges in disinhibited slices have longer durations than in induced slices (Fig. 7).

It should be noted that intermediate slices, where the neocortex immediately adjacent to the PC is transitional between AI and PRh (Fig. 1B), differed from both anterior and posterior slices in showing no onset in adjacent neocortex. Both disinhibited (n = 11) and induced (n = 11) slices of intermediate PC showed the same site of onset in the dorsal portion of the En, with very little involvement of adjacent AI/PRh.

Figure 13A shows that stimulation of an induced slice at different sites evoked discharges with approximately the same site of onset. Figure 13B shows that a similar site of onset is seen for discharges evoked by repeated stimulation of a disinhibited slice at the same site (the variations in onset site will be discussed later). The results from many experiments under each condition examined are summarized in Fig. 14. Contour maps show the sites of onset under disinhibited and induced conditions in PC slices from anterior (Fig. 14A), intermediate (Fig. 14B), and posterior (Fig. 14C) planes, to display the aggregate behavior and compare the results for different conditions. These locations are also summarized in Table 1.

Variation in site of onset

In general, the site of onset varied by as much as 150–300 μm (1–2 detectors) for different sites of stimulation...
FIG. 9. Overlays of fluorescent traces and video images above (as in Fig. 5) provide a reference for the intensity maps of this figure. Each trace in the overlay is 967 ms in duration. A: control intermediate PC slice was stimulated in layer Ib with 400 µA (single trial). B: induced intermediate PC slice was stimulated in layer II at 275 µA (single trial). Maps of fluorescence intensity encoded as color from the corresponding overlays above show spreading electrical activity. Color bars show the color scale with dark purple corresponding to lowest fluorescence and red corresponding to highest fluorescence. First frame shown was that immediately before the stimulus. Sequence of maps shows the spread of signals away from the site of stimulation (beginning in frame 2). In the control experiment (A), the responses are confined to the vicinity of the stimulus, and these responses last only until about frame 14. In the induced slice (B), the initial response at the site of stimulation (frames 2–14) is followed by the epileptiform discharge, appearing in frame 24 at the site of onset. In this experiment, the site of onset was the dorsal part of the En. Epileptiform activity originated at this site and spread to the overlying PC and the neighboring AI/PRh (frames 24–36). Each map shows a snapshot at 3.8-ms intervals.

(Fig. 13A), and this was similar to the variations seen from trial to trial for stimulation at a given site (Fig. 13B). The variability in site of onset for different slices in each condition studied here is indicated in Fig. 14.

Under induced conditions, one of eight anterior slices showed a site of onset located in the central rather than the dorsal part of the En, with no contribution from deep AI. Within this group, the site of onset generally included the transitional region between En and AI, with variable contributions from the dorsal part of the En and layer VI of the adjacent deep AI. Figure 14A, left panel, displays the sites of onset for six different experiments. In posterior induced slices, occasional variations in the site of onset also were observed. Four of 18 slices showed onset in the ventral or central parts of the En rather than the dorsal part observed more typically (Fig. 14C, left). In these four slices the site of onset did not include the deep PRh. Among the remaining 14 slices, 1 slice showed onset only on the dorsal side of the En, 2 showed onset only in layer VI of the PRh, and the other 11 slices showed discharge onset in both the dorsal part of the En and layer VI of the PRh. In 1 of 11 intermediate induced slices, the site of onset was on the ventral side of the En, whereas in the remaining 10 slices discharges originated in the dorsal side of the En (Fig. 14B, left). The small number of slices with atypical sites of onset is difficult to evaluate without examining a much larger sample. However, the site of onset appeared generally to be less variable in disinhibited slices than in induced slices (compare Fig. 14, left with right).
FIG. 10. Site of onset in anterior slices. A shows an induced slice and B a disinhibited slice. A1 and B1: Nissl-stained slides prepared after recording show the anatomic structures. OT, olfactory tubercle. For other abbreviations, see Fig. 1. Note the different numbering of cortical layers in A1. Left arrowhead marks the border of the PC and OT, and the right arrowhead marks the border of the PC and AI (in A1). A2 and B2: sites of discharge onset are shown as contours around the photodiode detector fields where the signal had surpassed 50% of the maximum change in fluorescence. In the induced slice, a stimulus current of 250 μA was applied to layer Ib. Site of onset included both the dorsal part of the En and part of layer VI of the adjacent AI (A2). In the disinhibited slice, a stimulus current of 95 μA was applied to layer III. Site of onset was located in the En (B2). Stimulus sites are indicated by ◊◊◊.

FIG. 11. Site of onset in posterior slices. A shows an induced slice and B a disinhibited slice. A1 and B1: Nissl-stained slides prepared after recording show the anatomic structures. Arrowheads mark the border between PC and PRh (see Fig. 1 for definitions of labels). Note the different numbering of cortical layers in PRh. A2 and B2 show contours as in Fig. 10 around detector fields where the signals were within 70% of the maximum fluorescence. In the induced slice in A, the site of discharge onset consisted not only of the dorsal part of the En but also part of layer VI of the PRh. In the disinhibited slice, the site of discharge onset in B was confined to the En. Slice in A2 was stimulated in layer Ib with 500 μA. Slice in B2 was stimulated in layer Ib with 140 μA. Stimulus sites are indicated by ◊◊◊.
The size of the onset region thus provides a rough indication about the number of neurons that must participate to initiate a discharge. The size of the onset region is difficult to gauge because the process is not stationary; the epileptiform discharge is spreading even as it begins. Thus the onset region could be smaller than the region in the earliest frame showing the discharge. Furthermore, there is variation in the size of the onset region that appears to be correlated with stimulus strength and latency (data not shown). Contours such as those in Figs. 10, 11, and 13 often had diameters as small as 300 μm (i.e., containing 4 photodiode detector fields). This can be taken as an upper bound to the size of a region where a discharge begins.

**Propagation velocity**

After initiation, epileptiform discharges propagated outward from the site of onset toward the overlying PC (Figs. 7–9). Data such as that shown in Fig. 8 can be used to measure the velocity of this process. The propagation velocity of epileptiform activity has been measured in a variety of cortical slice preparations (Albowitz and Kuhnt 1991; Albowitz et al. 1990; Chervin et al. 1988; Colom and Saggau 1994; Hoffman and Haberly 1993; Miles et al. 1988; Sutor et al. 1994) as well as in intact cortex (Goldensohn and Salazar 1986; London et al. 1989). Many of these studies reported velocities below the slowest action potential conduction velocities, and this difference has been put forward as support for a mechanism of propagation involving recurrent excitation (Hoffman and Haberly 1993; Miles et al. 1988). More generally, a slower velocity implies a greater role either for recurrent excitation or for other processes not directly related to axonal propagation such as ephaptic or electrotonic coupling (Dudek et al. 1986). We therefore measured propagation velocity under different conditions and in different directions.

Propagation velocity was determined by taking the time at which fluorescence was half-maximal and plotting the distance from the site of onset versus this time (Fig. 15). In the plot shown, data from induced slices from six animals are pooled for epileptiform discharges propagating from the sites of onset in the En to the overlying PC. With a threshold stimulus the propagation velocity was 0.101 ± 0.003 (SD) m/s. Velocities for propagation of nonepileptiform activity in control slices also were measured. Propagation was examined in control, disinhibited and induced slices in three directions as indicated in the diagram above Table 2. To evoke epileptiform discharges originating in layer II of the PC and spreading toward En, high suprathreshold stimulus currents were used. With sufficiently high stimulus strength the latency goes to zero (Fig. 6B), and the discharge originates near the site of stimulation.

Table 2 shows velocities for propagation in three directions, from En to PC, from PC to En, and in layer I along a pathway parallel to the cortical surface. In control slices, evoked voltage changes propagated with a velocity ranging from 0.16 to 0.22 m/s. This is in the lower range of action potential propagation velocities in unmyelinated axons (Eccles et al. 1967; Freeman 1972; Manis 1989). Propagation was slightly faster in layer I and slightly slower from En to PC.

We found that epileptiform activity generally propagated more slowly than action potentials, as noted previously by others (Hoffman and Haberly 1993; Miles et al. 1988). Discharges evoked in induced slices by high-stimulus currents propagated within layer I with velocities comparable with the velocities of action potentials in control slices, implying that action potentials could underlie discharge spread along this pathway. In contrast, epileptiform bursts in disinhibited slices propagated more slowly than action potentials regardless of direction and stimulus current. This then represents another difference between disinhibited and induced slices and suggests that when GABA<sub>A</sub> receptors are fully functional, epileptiform activity spreads within layer I by a different mechanism in which action potential conduction in axons could play a greater role.

**Discussion**

The present study shows that imaging of voltage-sensitive dye fluorescence is a powerful technique in the study of epileptiform activity in brain slices. As in previous studies (Albowitz and Kuhnt 1991; Albowitz et al. 1990; Colom and Saggau 1994; London et al. 1989; Sutor et al. 1994; Hoffman and Haberly 1993; Miles et al. 1988; Sutor et al. 1994) as well as in intact cortex (Goldensohn and Salazar 1986; London et al. 1989). Many of these studies reported velocities below the slowest action potential conduction velocities, and this difference has been put forward as support for a mechanism of propagation involving recurrent excitation (Hoffman and Haberly 1993; Miles et al. 1988). More generally, a slower velocity implies a greater role either for recurrent excitation or for other processes not directly related to axonal propagation such as ephaptic or electrotonic coupling (Dudek et al. 1986). We therefore measured propagation velocity under different conditions and in different directions.
FIG. 13. Site of onset of epileptiform discharges in an induced (A) and a disinhibited (B) intermediate slice. A1 and B1: Nissl-stained slices prepared after recording show the anatomic structures. Arrowheads mark the border between PC and AI/PRh. See Fig. 1 for definitions of labels. Note the different numbering of cortical layers in AI/PRh. A: contours representing 50% of maximum fluorescence change show the site of onset for different stimulation sites, indicated by different colors: green, stimulation in layer Ib with 55 μA; red, stimulation in layer III with 75 μA; blue, stimulation in layer III with 40 μA. B: contours representing 70% of maximum fluorescence show the site of onset for epileptiform discharges evoked 4 times by stimulation at the same site with currents slightly above threshold (range 29–34 μA). Different colored contours show how the site varied between trials. Sites of stimulus are indicated by .

1994 , this technique revealed the spatiotemporal pattern of spreading electrical activity, permitting localization of the site of onset of epileptiform activity and quantitative measurements of propagation velocity. The epileptiform activity reported here shows the same duration, latency, and all-or-none character as revealed by microelectrode techniques in the same preparation (Hoffman and Haberly 1989, 1991). Furthermore, the pharmacological properties of electrically evoked fluorescent dye signals are consistent with microelectrode studies (Haberly 1998). These similarities with microelectrode recordings and the correspondence between simultaneous optical and extracellular recordings (Fig. 4) demonstrate that voltage-sensitive dye fluorescence reports electrical activity with high fidelity, thus validating the use of optical signals in the study of epileptiform behavior in slices from the PC.

Site of origin

Early experiments in vivo suggested that seizures could be evoked by injection of convulsants in the deep PC (Piredda and Gale 1985; Stevens et al. 1988). Subsequent work in vitro identified the En as well as the claustrum and deep layer III of the PC as sites of high seizure susceptibility (Hoffman and Haberly 1991). Our work confirms the finding that the site of onset is in the dorsal part of the En and that a slow build-up of electrical activity occurs at that site (Hoffman and Haberly 1993). Further, we have shown here...
ONSET OF EPILEPTIFORM DISCHARGES IN PIRIFORM CORTEX

FIG. 15. Distance is plotted vs. time for the spread of a threshold epileptiform discharge. Time is taken when the fluorescence is half-maximum in the rising phase. Slope of this plot gives the velocity of discharge propagation as $0.101 \pm 0.003 \text{ m/s}$. Data are from experiments in 5 induced intermediate slices and 1 induced posterior slice, with threshold stimulus currents applied to either En, layer III, or layer Ib. Stimulus current ranged from 15 to 400 $\mu$A in the 6 experiments. For velocities under various conditions, see Table 2.

En. Thus the adjacent neocortex was a site of origin in induced anterior and posterior slices. By contrast, in disinhibited slices epileptiform discharges originated only in the En for slices along the entire anterior-posterior axis.

Previous studies have shown that epileptiform discharges can be triggered by injection of glutamate or $K^+$ into the claustrum (Hoffman and Haberly 1991), but when we stimulated diverse locations throughout the PC, epileptiform discharges consistently originated in the En and deep AI in slices of anterior PC and in the En in slices of intermediate PC. These slices contained claustrum (Fig. 1), but discharges were never seen to originate there. Thus although the claustrum has a high seizure susceptibility, the fact that the onset occurred in the En and deep AI indicates that the seizure susceptibility of these structures surpasses that of the claustrum. Our observation of simultaneous onset in the adjacent PRh, may be relevant to the involvement of the perirhinal cortex in kindling (McIntyre and Plant 1993). However, those studies implicated perirhinal cortex in a later stage of kindling rather than onset, as found here.

The highly characteristic site of onset in PC slices stands in striking contrast with the results of similar experiments in hippocampal slices (Colom and Saggau 1994). In these studies, the site of onset varied considerably over the CA2 and CA3 fields. Even under a given condition variability was evident, and additional variations were associated with conditions and species. Thus the hippocampus does not appear to have a clearly defined hot spot for discharge generation comparable to the En of the PC.

The present results on PC slices direct our attention to small, well-defined locations deep in the piriform region. Further study to determine the cellular properties and circuitry in these locations may indicate what factors lead to high seizure susceptibility. In this regard, it is relevant that

![Fig. 14. Summary of sites of onset. Contours marking the sites of discharge onset as in Figs. 10, 11, and 13 were drawn over sketches of slices from anterior, intermediate, and posterior PC for the 2 in vitro models used in this study. Each sketch contains contours from 6 experiments, except for disinhibited-posterior for which 5 experiments were used. These results are also summarized in Table 1. See Fig. 1 for definitions of labels.]

That there are additional sites of onset in the induced model and that in the disinhibited model epileptiform discharges show similar but not identical sites of onset. Only in intermediate PC slices did epileptiform discharges originate in the dorsal part of the En for both models. In induced slices of anterior PC, epileptiform discharges originated in both the dorsal portion of the En and layer VI of the adjacent AI. In induced slices of posterior PC, discharges originated in layer VI of the adjacent PRh, along with the dorsal portion of the

![Table 1. Sites of discharge onset]

This table summarizes the aggregate results shown in Figs. 10, 11, and 13 on the site of discharge onset. For discussion of variability in site of onset, see text. PC, piriform cortex; En, endopiriform nucleus; AI, agranular insular cortex; PRh, anterior perirhinal cortex.
Table 2. Propagation velocities

<table>
<thead>
<tr>
<th></th>
<th>En → PC (1)</th>
<th>PC → En (2)</th>
<th>Layer I (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 100 μA</td>
<td>0.16 ± 0.01 (4)</td>
<td>0.20 ± 0.01 (4)</td>
<td>0.19 ± 0.02 (4)</td>
</tr>
<tr>
<td>Control 400 μA</td>
<td>0.18 ± 0.01 (4)</td>
<td>0.19 ± 0.01 (4)</td>
<td>0.22 ± 0.01 (4)</td>
</tr>
<tr>
<td>Disinhibited 100 μA</td>
<td>0.092 ± 0.004 (5)</td>
<td>0.095 ± 0.004 (4)</td>
<td>0.080 ± 0.005 (3)</td>
</tr>
<tr>
<td>Disinhibited 400 μA</td>
<td>0.108 ± 0.004 (5)</td>
<td>0.112 ± 0.005 (5)</td>
<td>0.114 ± 0.006 (4)</td>
</tr>
<tr>
<td>Induced 100 μA</td>
<td>0.106 ± 0.003 (6)</td>
<td>0.14 ± 0.01 (6)</td>
<td>0.15 ± 0.01 (5)</td>
</tr>
<tr>
<td>Induced 400 μA</td>
<td>0.111 ± 0.005 (6)</td>
<td>0.12 ± 0.01 (6)</td>
<td>0.20 ± 0.02 (5)</td>
</tr>
<tr>
<td>Threshold</td>
<td>0.101 ± 0.003 (6)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Velocities were measured as the slope in distance versus time plots (Fig. 15) along the various axes shown in the schematic above. The velocities for control activity reflect action potential conduction velocities. The velocities in disinhibited and induced slices reflect the velocities for propagation of epileptiform discharges. See Fig. 1 for the definitions of abbreviations used in the sketch above. Values are means ± SD with number of slices in parentheses.

Different in vitro models

Although the two different in vitro models of epilepsy employed here gave qualitatively similar results, a number of subtle differences were seen. In disinhibited slices epileptiform discharges lasted longer than in induced slices. The most likely explanation for this difference is that discharges terminate by different mechanisms in the two models. In disinhibited slices GABA<sub>A</sub> receptors are blocked so that the release of GABA will be less effective in discharge termination. Inhibitory synaptic potentials are thought to play a role in discharge termination (Matsumoto and Ajmone-Mansan 1964), and it is likely that the shorter duration of discharges in induced slices is caused by the activation of GABA<sub>A</sub> receptors at an early stage of the burst. In contrast, when slices have been disinhibited by a GABA<sub>A</sub> receptor antagonist, another form of slow negative feedback may be engaged to terminate epileptiform discharges. One possible mechanism is the activation of Ca<sup>2+</sup>-activated K<sup>+</sup> channels as Ca<sup>2+</sup> accumulates within bursting neurons (Alger and Williamson 1988; Silva-Barrat and Champagnat 1995; Wong and Prince 1981). A Ca<sup>2+</sup>-activated K<sup>+</sup> current is present in both superficial pyramidal cells and deep multipolar spiny cells of the piriform cortex, but this current is stronger in deep cells (Tseng and Haberly 1989b). Another possibility for discharge termination in disinhibited slices includes the activation of bicuculline-resistant inhibitory receptors. A slow inhibitory synaptic potential mediated by GABA<sub>B</sub> receptors has been described in this preparation (Tseng and Haberly 1988). Finally, discharge termination also may depend on desensitization of excitatory amino acid receptors, inactivation of voltage-gated Na<sup>+</sup> and Ca<sup>2+</sup> channels, and K<sup>+</sup> accumulation in the extracellular space.

The two in vitro models employed here also differ in their site of discharge onset. In the induction model, layer VI of the AI and PRh, were additional sites of onset in anterior and posterior PC slices, respectively. Epileptiform bursts arise at these sites at the same time as in the dorsal part of the En, which is the only site of onset in disinhibited slices. This observation is relevant to the discussion above on the role of cell properties, cell density, and synaptic connectivity in determining the site of onset. The low seizure threshold of deep somatosensory cortex has been attributed to endogenously bursting neurons (Agmon and Connors 1989; Chag-Hac-Amitai and Connors 1989; Connors 1984). The distributions of endogenously bursting neurons, deep multipolar cells, and calretinin-containing interneurons (Domroese et al. 1997) in the sites of onset within the deep piriform region and adjacent neocortex could lead to discharge generation by different mechanisms in different in vitro models. For example, as indicated earlier, low-cl<sup>−</sup> induction may operate through the enhancement of excitability in calretinin-containing neurons distributed through both En and adjoining deep Al or PRh (Domroese et al. 1997). On the other hand, the En may have more interneurons not subject to inhibition by calretinin-containing interneurons, or more generally, GABA<sub>B</sub> receptors may play a greater role in limiting electri-
cal activity in the En so that blockade of GABA_A receptors would have a greater effect in this location. However, the simultaneous onset of epileptiform discharges in En and Al or En and PrH in induced slices almost certainly will depend on the strong reciprocal excitatory connections between these structures (Beckstead 1979; Datiche and Cattarelli 1996; Reep and Winans 1982).

Modes of propagation

Epileptiform activity spreads too slowly to be explained solely by action potential conduction (Hoffman and Haberly 1993; Miles et al. 1988). This was confirmed in the present study and shown to hold for discharge propagation in various directions under various conditions. Possible mechanisms consistent with slower conduction velocities include regenerative positive feedback through recurrent excitatory synapses, ephaptic interactions, and electrotonic coupling (Dudek et al. 1986). Variations in velocities of spread along different trajectories could mean that these mechanisms make different contributions to the spread of epileptiform activity in different regions. Furthermore in layer I of induced slices, epileptiform activity spreads with a velocity close to that of the slowest action potentials. This may mean that action potential conduction along the dense association fibers in this layer plays a greater role.

We thank K. Manning for assistance in photographing histology sections. This research was supported by National Institute of Neurological Disorders and Stroke Grants NS-19865 to L. B. Haberly and NS-37212 to M. B. Jackson. Grants from the Wisconsin Alumni Research Foundation and from the University of Wisconsin Medical School Research Committee to M. B. Jackson also provided support. R. Demir was supported by a fellowship from the American Association of University Women.

Address for reprint requests: M. Jackson, Dept. of Physiology, SMI 129, University of Wisconsin Medical School, 1500 University Ave., Madison, WI 53706.

Received 23 April 1998; accepted in final form 20 July 1998.

REFERENCES


