Effects of the Gliotoxin Fluorocitrate on Spreading Depression and Glial Membrane Potential in Rat Brain In Situ

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Largo, Carlota, José M. Ibarz, and Oscar Herreras. Effects of the gliotoxin fluorocitrate on spreading depression and glial membrane potential in rat brain in situ. J. Neurophysiol. 78: 295–307, 1997. DC extracellular potential shifts (ΔV_o) associated with spreading depression (SD) reflect massive cell depolarization, but their cellular generators remain obscure. We have recently reported that the glial specific metabolic poison fluorocitrate (FC) delivered by microdialysis in situ caused a rapid impairment of glial function followed some hours later by loss of neuronal electrogenic activity and neuron death. We have used the windows for selective decay of cell types so created to study the relative participation of glia and neurons in SD, and we report a detailed analysis of the effects of FC on evoked SD waves and glial membrane potential (V_o). Extracellular potential (V_e), interstitial potassium concentration ([K^+]_i), evoked potentials, and transmembrane glial potentials were monitored in the CA1 area before, during, and after administration of FC with or without elevated K^+ concentration in the dialysate. SD waves propagated faster and lasted longer during FC treatment. ΔV_o in stratum pyramidale, which normally are much shorter and of smaller amplitude than those in stratum radiatum, expanded during FC treatment to match those in stratum radiatum. The coalescing SD waves that develop late during prolonged high-K^+ dialysis and are typically limited to stratum radiatum, also expanded into stratum pyramidale under the influence of FC. SD provoked in neocortex normally does not spread to the CA1, but during FC treatment it readily reached CA1 via entorhinal cortex. Once neuronal function began to deteriorate, SD waves became smaller and slower, and eventually failed to enter the region around the FC source. Slow, moderately negative ΔV_o that mirrored [K^+]_i increments could still be recorded well after neuronal function and SD-associated V_o had disappeared. Glial cell V_o gradually depolarized during FC administration, beginning much before depression of neuronal antidromic action potentials. Calculations based on the results predict a large decrease in glial potassium content during FC treatment. The results are compatible with neurons being the major generator of the ΔV_o associated with SD. We conclude that energy shortage in glial cells makes brain tissue more susceptible to SD and therefore it may increase the risk of neuron damage.

INTRODUCTION

Neuron injury in the penumbra of an ischemic focus has been attributed to recurrent spreading depression (SD) (Iijima et al. 1992). Similarly, sustained SD-like depolarization caused by ischemia and anoxia, both in vitro and in situ, appears to damage neurons (Balestrino et al. 1989; Herreras and Somjen 1993c; Kawasaki et al. 1988). It is known that most brain insults that ultimately lead to neuronal death induce a rapid and lasting astrocyte swelling and extracellular acidosis (review by Kimelberg et al. 1990), a condition often associated with cell dysfunction. Recently, we found that specific deterioration of glial cells in situ caused by fluorocitrate (FC) was followed by neuron death a few hours later, and the demise of neurons appeared to depend on the passage of repeated SD waves (Largo et al. 1996). The experiments reported here continue previous work, and focus on the relative contribution of the different cell types, glia or neurons, to the generation of SD.

The biophysical basis of SD is incompletely understood. SD moves slowly through the brain accompanied by a characteristic large sustained negative extracellular potential shift (ΔV_o) (for review, see Bureš et al. 1974; Marshall 1959; Nicholson and Kraig 1981). The ΔV_o and the neuronal membrane potential (V_m) and membrane resistance changes during severe hypoxia and terminal depolarization (TD) are very similar to those seen during SD (Czež et al. 1993; Hansen 1985; Marshall 1959). The generators of the SD-related ΔV_o have not definitely been identified (Somjen 1973; Wadman et al. 1992). The much smaller ΔV_o recorded during repetitive activation and during seizures are usually attributed to spatially distributed currents flowing through the glial electrotonic quasisynaptic network and returning through interstitial space (Dietzel et al. 1989; Gardner-Medwin 1983; Hertz 1965; Newman 1995; Orkand et al. 1966; Somjen 1973, 1978, 1995). The larger ΔV_o recorded during SD and hypoxia are associated with a massive depolarization of neurons as well as glial cells and a redistribution of ions, but the exact mechanism of their generation is not clear.

In previous experiments in rats in vivo we defined two major components of the SD-related ΔV_o waves that could be dissociated pharmacologically and that behaved differently with respect to ion shifts (Herreras and Somjen 1993a,b,d). However, the data did not identify the specific generators of the component V_o shifts. Most circumstantial evidence points to neurons as the main cells involved in SD (e.g., Herreras et al. 1994; Hull and Van Harreveld 1964), but glial cells have also been considered as the main cellular type supporting SD movement (Hertz 1965; Higashida et al. 1974; Sugaya et al. 1975). We have examined the contribution of glial cells to the generation of SD in situ by using the metabolic poison FC (Largo et al. 1996; Paulsen et al. 1987), a ‘suicide’ substrate for the enzyme aconitase that is selectively taken up by glial cells (Clarke et al. 1970). This drug and the related fluoroacetate have been used in several studies requiring temporary arrest of glial function (Berg-Johnsen et al. 1993; Hassel et al. 1995; Keysar and Pellmar 1994; Paulsen et al. 1987; Stone et al. 1990; Szerb 1991). In recent work we found that FC delivered by micro-
dialysis in the CA1 region causes early glial swelling and dysfunction before deterioration of neurons, a process that was accelerated by the spontaneous occurrence of SD waves, allowing a suitable time window to separate the role of the two cell types. Some of the results reported here have been presented in abstract form (Herreras et al. 1996).

METHODS

Preparation and recording

Experiments were performed in the dorsal CA1 region of urethane-anesthetized (1.2 g/kg ip) female Sprague-Dawley rats weighing 200–250 g. Body temperature was maintained at 37 ± 0.1°C with a heating blanket. Surgery and stereotaxic procedures were carried out as previously described in detail (Herreras 1990; Largo et al. 1996). A concentric bipolar stimulating electrode was placed in the alveus for antidromic activation, or in the ipsilateral CA3 for orthodromic activation of the CA1 field. Recording electrodes were glass micropipettes filled with 150 mM NaCl (5–10 MΩ). Pipettes were usually placed at the cell body layer of CA1 field and/or in the stratum radiatum, guided by the typical evoked field potentials. They were sometimes used in vertical arrays of two to four pipettes glued so as to record from different depths at essentially the same anterior and lateral coordinates. These arrays were constructed and used as previously described (Herreras and Somjen 1993b).

The Ag/AgCl wires of recording micropipettes were connected to DC-coupled input stages. A subcutaneous Ag/AgCl wire electrode located under the neck skin of the animal served as reference. After amplification, DC signals were stored on a video cassette recorder and processed off-line by computer programs after acquisition at sampling rates of either 5–20 Hz or 5–10 kHz.

Double-barreled ion-selective microelectrodes were manufactured and used as in previous reports (Herreras and Somjen 1993b; Largo et al. 1996). The Fluka ion exchanger No. 60031 or the WPI cocktail IE-190 was used as liquid membrane for K⁺. The reference barrel served for the recording of evoked potentials and DC voltages. The initial control level of interstitial potassium concentration ([K⁺]i) could not always be reliably determined relative to a calibration solution bathing the cortex because of spurious sources for DC potential as the electrode was inserted into the tissue. In these cases it was assumed that the resting [K⁺]i was 3.4 mM.

Microdialysis

Microdialysis probes (220 μm OD, length 0.8–1 mm) manufactured as previously described (Herreras et al. 1989, 1994) were used to deliver the FC and/or high-K⁺ solution. Before the probe was inserted into the brain a micropipette electrode was introduced to find an appropriate location, guided by recording the evoked potential. Then the probe was lowered to the same position so that the entire dorsoventral extension of the CA1 region was exposed to dialysis. The location was A = −4 to −5.5, L 2.6–3. Insertion of the dialysis probe invariably provoked a single SD wave, recorded by a nearby micropipette.

Control artificial cerebrospinal fluid (ACSF) had the following composition (mm): 122 NaCl, 3 KCl, 0.4 KH2PO4, 1.2 CaCl2, 1.2 MgSO4, and 25 NaHCO3. A peristaltic pump was used at a constant rate of 2 μl/min. To provoke SD, 100 mM KCl (high-K⁺) was substituted for NaCl. FC was dialyzed at a concentration of 1 mM, which is ~10 times higher in the perfusate than that required to exert the desired effect in the bathing fluid of in vitro preparations (Berg-Johnsen et al. 1993; Herreras and Somjen 1993a,b; Herreras et al. 1989; Largo et al. 1996). The actual concentration of drugs at specific distances from the dialysis fiber could not be determined.

Experimental protocol

In previous experiments (Largo et al. 1996) FC was perfused by itself. In the present study we analyzed the early changes that were presumably caused by impairment of glial function and that occurred before any SD waves were observed. A 90-min rest period was allowed after the probe implantation before the experiment was started; then high-K⁺ ACSF was perfused for 5 min to elicit a control SD. Thirty minutes later, FC was perfused for varying times. In a group of experiments, either a high-K⁺ ACSF plus FC (n = 3) or high-K⁺ alone (n = 3) were perfused for 3 h to study changes in the evolution of the SD waveform. As reported (Herreras and Somjen 1993a,b), SD waves originated at the source of high-K⁺ and repeated one after another in a consistent pattern, spreading throughout the CA1 region. In another series (n = 4), only FC was perfused for 8 h and one SD was elicited each hour by microinjection of KCl (see below). One more series (n = 4) was used to study the evolution of spontaneous SD waveforms and potassium transients at late times of FC perfusion alone (8 h). This series was pooled with previous experiments (n = 12) used to measure other variables (Largo et al. 1996). The passage of SD waves between cortex and CA1 was studied in three additional animals dialyzed with FC for 6–8 h, during which SD was initiated at different positions by KCl ejection.

Pressure ejection of solutions

SD waves were sometimes elicited by pressure ejection of 1.2 M KCl from a glass micropipette (2–5 μm at the tip) located ~100 μm below the pyramidal cell layer. This position showed the lowest threshold to evoke SD (Herreras and Somjen 1993b). The ejected volume was estimated outside the tissue, ranging from 25 to 50 nl. The same pipettes also served for electrical recording, enabling the monitoring of evoked potentials during insertion for the accurate placement of the pipettes and subsequently the registration of the voltages at the site of SD initiation or passage. When no pressure was applied, a stable DC baseline and normal evoked potentials recorded from the high-K⁺ pipettes indicated that leak of K⁺ was negligible or readily buffered by surrounding cells and/or by diffusion.

Intracellular recordings

Cells were impaled by glass micropipettes backfilled with 3 M potassium acetate (60–120 MΩ). Recordings were amplified, filtered at 10 kHz, and stored on a video cassette recorder. Impalements were made within <1 mm from the dialysis surface before and during FC delivery. Exact distance cannot be accurately determined because of technical constraints. Dialysis probes were bent twice to allow room for the intracellular pipette and holding device. The active surface was 800–900 μm, extending from the alveus to the hippocampal fissure. Glial cells were identified by the absence of injury discharge on impalement, high resting Vm (75–90 mV), absence of synaptic potentials and action potentials, and slow depolarizing potentials in response to nearby neural activity (Gutnick et al. 1981). Later during FC treatment, resting Vm could not be used as a reliable feature, but safe identification was still possible on the basis of the remaining criteria. The voltage recorded on withdrawal from the cell was used as the reference to calculate transmembrane potentials. The micropipette was advanced by a motor-driven piezomanipulator continuously performing vertical tracks from the cortex surface to beyond the hippo-
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Campal fissure. Only glial cells located within 1 mm of the cell body layer were used for this study, spanning the entire CA1 and 700 and 500 μm above (cortex) and below (fascia dentata), respectively. The stratum pyramidale was identified by the evoked antidromic population spike (a-PS), which can be recorded unchanged even after several hours of FC perfusion (Largo et al. 1996). The depth was determined relative either to the stratum pyramidale or to the cortical surface.

Statistics

The effect of FC on glial $V_o$ was analyzed by a multivariate linear regression. The maximum model included perfusion time, position, and treatment (ACSF or FC), and the interactions of the latter with the former two. Treatment was codified as 0 for absence of FC (control) or 1 for presence (experimental). Because the FC was delivered only to the CA1 and cells were also impaled in cortex and fascia dentata, where FC can also reach by diffusion, the relevance of the position was studied by considering three gross locations—CA1, cortex, and fascia dentata—and creating two "dummy" variables for the latter two. Nonsignificant variables were sequentially dropped from the model, and the value of the partial $F$ was used to calculate the significance between correlated variables (significance level was $\alpha = 0.05$). The final model contained the variables time, treatment, and their interaction, and was highly significant ($P < 0.001$). Although linear regression was chosen to allow the global analysis of all variables, nonlinear regressions may as well be possible and therefore no conclusions will be drawn as to the linearity of the relations. Partial comparisons were also made by using Student’s $t$-test ($\alpha = 0.01$). Significance of the partial linear regressions was studied with the use of the probability of the $F$ value ($\alpha = 0.01$).

RESULTS

FC administration fosters SD waves

The effects of FC on SD waveforms elicited by continuous $K^+$ perfusion are shown in Fig. 1. The top row illustrates the main features of SD-associated $\Delta V_o$ in the control state, from an experiment in which a vertical array of four pipettes was used in positions ranging from stratum pyramidale to stratum radiatum. The $\Delta V_o$ correspond to two semi-independent SD waves running in parallel through the apical dendritic tree (thin tracings) and the stratum pyramidale (thick tracings) (see Herreras and Somjen 1993a,b). In both layers the $\Delta V_o$ consist of an early fast and a later slow component, labeled $a$ and $b$ in Fig. 1. The duration of the dendritic $\Delta V_o$ increased with repetition and the amplitude reached a maximum after four to six waves because of the growth of the slow component. The somatic $\Delta V_o$ are much shorter and smaller, and changed little with repetition. Unlike in control condition, shortly after the start of FC administration the high-$K^+$-induced, SD-associated $\Delta V_o$ waves in stratum pyramidale began to grow and rapidly approached in amplitude where SD waves could be recorded at a shorter latency than in stratum radiatum that were typically preceded by up to 40 s by small (3–5 mV), slow, positive, biphasic or triphasic voltage deflections (Fig. 3, bottom, *). To explore the origin of this signal, we used a roving pipette to eject high-$K^+$ solution, in addition to a NaCl-filled recording pipette whose location remained fixed in CA1 stratum pyramidale (see Fig. 3, top). In the absence of FC, microinjection of $K^+$ into neocortex provoked an SD-like $\Delta V_o$ recorded by the cortical electrode, which was accompanied by a small triphasic wave in CA1, but not SD in CA1. After the roving electrode was lowered into stratum radiatum, a similar injection provoked, as expected, SD at the injection site that then propagated to the other recording electrode in stratum pyramidale (Fig. 3). Later in the same experiment during FC dialysis, after the roving pipette was repositioned in neocortex, a similar $K^+$ injection in neocortex provoked SD accompanied by the small biphasic wave in stratum pyramidale, which, however, was now followed by SD. This corticohippocampal invasion was repeated in 9 of 11 trials in three experiments while FC was perfused by itself. The small DC deflections arose in all cases. In two of the experiments, the CA1 electrode was moved caudally toward subiculum and entorhinal cortex, where SD waves could be recorded at a shorter latency than in CA1 after cortical KCl injection. It appears that the SD provoked in neocortex was, during FC administration, able to spread into hippocampus via entorhinal cortex and subiculum.

On three occasions, when SD was provoked in the caudal portions of the cortex, a prolonged negative drift of the baseline occurred that could be recorded in both neocortex and hippocampus (– – – in Fig. 3).
FIG. 1. Effect of fluorocitrate (FC) perfusion on evolution of spreading depression (SD) waveforms at different strata. SD waves arose from vicinity of dialysis fiber perfusing 100 mM KCl. **Top row:** simultaneous recording of SD waves by vertical array of 4 pipettes. SD was much shorter in stratum pyramidale (thick tracings), whereas that in stratum radiatum lengthened to maximum after several episodes. **a** and **b:** fast and slow components, respectively. **Bottom row:** in another experiment in which 2 pipettes were used, duration of SD increased during FC perfusion (note different time scaling). SD in stratum pyramidale gradually became longer, adopting waveform similar to that in stratum radiatum. **Insets:** characteristic orthodromically evoked potentials recorded at corresponding strata (distance from stratum pyramidale). Dashed tracings were recorded after 1 h of FC plus KCl perfusion. Dots: stimulus artifact (this and subsequent figures). Numerals above SD waveforms: elapsed time after KCl or KCl plus FC dialysis began.

**Late decline of SD during FC perfusion**

We have previously reported that during prolonged FC administration, when the amplitude of the a-PS decreases, the $\Delta V_o$ of SD waves also become depressed (Largo et al. 1996). To study in detail the gradual changes of SD waveforms, we elicited SD waves by microejection of high-$K^+$ at different times of FC dialysis in four animals. In these trials potassium was not perfused through the dialysis probe to avoid possible damage due to prolonged continuous exposure to elevated $[K^+]_o$ (Herreras and Somjen 1993c). As illustrated for a typical experiment in Fig. 4, 1 h after FC perfusion began, while the a-PS was still intact, the $\Delta V_o$ of provoked SD waves (Fig. 4A) were much longer and moved faster (~120%). After an average of 4–6 h of FC dialysis, when the a-PS amplitude began to decline (see Fig. 5, insets), the $\Delta V_o$ near the dialysis probe were strongly depressed and at the other two recording sites the main component $b$ was shorter and smaller (Fig. 4A, arrows). Moreover, the speed of propagation decreased to about half the initial value (~57%). At even later times SD waves did not enter at all the region closer to the probe.

Similar results were observed for the spontaneous SD waves that appeared after 3–6 h of FC perfusion (without high-$K^+$ in the dialysis fluid). Figure 4B shows that sample SD waves from the same experiment as in Fig. 4A, probably originating in the neocortex as suggested by the preceding small positive DC deflections, entered the hippocampus from the caudal extremity (thin trace) and proceeded rostralward (thick tracings and ——). These spontaneous SD waves, which repeated at regular intervals of 14–18 min for 2–3 h (e.g., Fig. 5), had an initial duration similar to those elic-
FIG. 2. Under influence of FC, coalescing SD waves extend into stratum pyramidale (sp). Top: occurrence of series of coalesced SD waves in stratum radiatum (sr). Characteristic positive hump of SD waves (†) was absent when somatic SD failed in all but 1st SD wave, adopting squarelike pattern (∗2). Occasionally, positive DC excursions were observed (∗1) due to aborted recovery of negativity. Bottom: loss of refractoriness in stratum pyramidale in series of coalesced SD waves during glia poisoning. Note positive hump in slower component of each SD wave (†). Insets: evoked potentials obtained before KCl or KCl plus FC dialysis began at indicated depth.

One more concomitant of the progressive decrease of the SD-associated ΔVₒ and [K⁺]ₒ transients was the abolition of the typical post-SD undershoot of [K⁺]ₒ. Figure 6 illustrates this effect. The undershoot of [K⁺]ₒ at the termination of the SD wave was replaced by a long tail above the pre-SD level that seems to contribute to the buildup of the baseline [K⁺]ₒ as illustrated in Fig. 5.

TD

Previously we have reported that the long-lasting negative displacement of Vₒ that occurs after cardiac arrest known as TD was absent in a region where glia as well as neurons appeared to have succumbed to prolonged treatment by FC (Largo et al. 1996). Figure 7 illustrates a more detailed analysis of this phenomenon. Shortly after cardiac arrest, an initial fast negative ΔVₒ marks the beginning of TD (Fig. 7A, ∗) that is followed by a prolonged slower negativity. In a region out of the reach of perfused FC (thin tracing), the maximum voltage of this initial phase was similar to a preceding SD wave elicited just before cardiac arrest. However, during recording within the core of the region treated with FC for 5 h (thick tracing), both the initial phase of TD and the preceding SD were similarly reduced (Fig. 7A, ↔). The final negative DC voltage corresponding to the slower

The gradual decrease of the ΔVₒ associated with SD was, however, delayed when compared with the decrease of their potassium transients (Fig. 5A). The SD-associated [K⁺]ₒ began to diminish already during the first few SD occurrences, whereas the ΔVₒ decline was not apparent until an acceleration in the increase of baseline [K⁺]ₒ occurred 1–2 h later (Fig. 5, †1). This baseline potassium accumulation was mirrored by a parallel slow negative extracellular potential (Vₒ). The DC baseline showed a very slow negative shift also during K⁺ dialysis after very prolonged FC treatment at a time when SD could no longer be elicited (Fig. 5, † 2). A distinct slow negative Vₒ of 8–12 mV with no potassium buildup occurred earlier (∗); it marked the beginning of the SD series. A similar negative shift could occasionally be recorded in the absence of FC, when a cortical SD wave was induced by K⁺ ejection and regardless of whether or not this invaded the CA1 (see Fig. 3).

SD waves usually continued unabated in neocortex while they became depressed in hippocampus (Fig. 5), indicating that the depression is specific to the region where FC is highly concentrated.
component of TD did not differ from that in untreated tissue when SD had not been completely abolished before death or when it had only been abolished for a short period (<1 h). Another experiment, where FC was perfused for 8 h and SD had already been abolished 90 min before death, is illustrated in Fig. 7B. Note the fast drop and maintained significant (t-test, \( P \leq 0.001 \)) the difference was not significant. The difference between each cell population during FC and ACSF perfusion (pooled) was highly significant (t-test, \( P \leq 0.001 \)). The fitted lines in Fig. 8C correspond to individual linear regressions, \( P \) values being only significant for CA1 (\( P \leq 0.001 \)).

Recordings of \( V_{m} < 30 \) mV, far more frequent after 3 h of FC perfusion, where discarded because it was not possible
FIG. 4. Failure of SD waves to invade vicinity of dialysis fiber after prolonged perfusion of FC. Left: arrangement of devices. All pipettes were located in stratum radiatum. Arrows and shading: direction of SD propagation. A: evolution of SD waves elicited by K⁺ microejection from pipette (inj) located rostral to dialysis fiber (d). After 1 h of FC perfusion, SD waves were longer at all locations and speed of SD had increased (small numerals: delay of SD between 2 caudal electrodes). After 5 h, speed had decreased below control, slower components were shorter (arrows), and amplitude of wave close to dialysis fiber was greatly diminished. DC baseline was more negative at caudal locations. B: in same experiment, spontaneous SD waves, probably originating in neocortex, invaded CA1 via entorhinal cortex (note entrance of SD wave at caudalmost electrode). With repetition, whether spontaneous or evoked, SD wave gradually decreased in amplitude and then failed to enter vicinity of dialysis fiber (thick tracings), where neurons are probably dead or injured. Note positive bump (**) before SD occurrence, during passage of SD wave in overlying cortex before its penetration in CA1. Delay between SD waves was measured between rostral and caudal electrodes. All recordings were made in stratum radiatum.

to discriminate between true glial impalements versus juxta-position potentials, spurious potentials, cells damaged by the electrode, or even some inactivated neurons. The elimination of very low \( V_m \) values may have biased the statistic because at least part of the rejected data must have been for glial cells depolarized by the FC treatment.

DISCUSSION

Biphasic effect of FC treatment

There was a striking difference in the effect of FC treatment during the initial few hours of FC dialysis, during which SD was facilitated, compared with later, when SD failed to invade the vicinity of the dialysis probe delivering FC. We know from the literature that energy shortage in glial cells is a major consequence of FC poisoning (Hassel et al. 1994), and only later causes degeneration of neurons (Largo et al. 1996; Paulsen et al. 1987). Temporal correlation suggests that the initial facilitation may be related to the impairment of glial function, whereas the eventual failure of the SD process may be the result of damage to the neuron population. We must now discuss whether these conclusions are justified.

The initial facilitation manifested itself in an acceleration of SD propagation and a marked expansion of the \( \Delta V_o \). Especially striking was the increase of the \( \Delta V_o \) recorded in stratum pyramidale, which became equal to that in stratum radiatum. Moreover, the prolonged, unstable SD state that normally spares stratum pyramidale now extended into this layer. These changes occurred while glial \( V_m \) began to de-
FIG. 5. Slow DC potentials and extracellular potassium during FC dialysis without elevated K⁺, except where indicated at end of tracing. Simultaneous recording with K⁺-sensitive electrode (e₁) in stratum pyramidale and micropipette (e₂) in overlying cortex, during long perfusion of FC. At ~3 h of FC administration, spontaneous cortical SD wave invaded CA1, leaving protracted negativity (X) in both regions. Interstitial potassium concentration ([K⁺]ᵢ) did not increase during this slow negative shift. After several SD episodes in CA1, K⁺ began to accumulate, and different slow negativity was added on already standing negative baseline (↑1). This remained as long as K⁺ was elevated, even after neurons were dead [note abolition of antidromic population spike (a-PS) in top insets]. Cortical SD waves continued for some time, although with slight decrease in amplitude after those in CA1 had disappeared. Perfusion of 125 mM KCl in CA1 caused additional K⁺ increase that was paralleled by slow negative potential (↑2). Box: arrangement of devices. S, stimulating electrode in alveus.

crease (Fig. 8). In previous work we found that morphological changes of astrocytes as well as failure of [K⁺]ᵢ and extracellular pH regulation began in the initial few hours of FC dialysis (Largo et al. 1996). There is much evidence that glial cells play an important role in the regulation of [K⁺]ᵢ and extracellular pH (Chesler 1990; Walz and Hertz 1983).

Unlike glial cells, neurons maintained their morphology as well as the capability to generate antidromically conducted action potentials for several hours of FC treatment (Largo et al. 1996; see also Berg-Johnsen et al. 1993; Keyser and Pellmar 1994; Stone et al. 1990). Neurons do not, however, completely escape subtle changes, because in some preparations orthodromic transmission can begin to decrease already within the 1st h of FC dialysis (Largo et al. 1996). The failure of synaptic transmission has, however, been considered to be a secondary consequence of deteriorating glial function (Keyser and Pellmar 1994; Largo et al. 1996).

The eventual failure of SD to invade the area surrounding the dialysis probe after prolonged FC treatment coincided in time and location with the failure of the antidromically conducted PS. It seems likely that the two failures are linked, and both are due to the deterioration of the neuron population. Injury to neurons may either be secondary to the loss
FIG. 7. Effect of FC perfusion on extracellular potential shifts (ΔV_o) during terminal depolarization (TD). A: simultaneous recordings obtained close to (thick tracings) and far from (thin tracings) dialysis probe delivering FC. SD potential, initially similar in both locations (left tracings) decreased earlier in vicinity of fiber. After cardiac arrest (c.a.) initial fast voltage drop of TD (●) behaved as last SD (●), although subsequent slower negativity was similar. Break in tracings is ~1 h. B: in another experiment, when SD had disappeared completely before death, closer K⁺ electrode (V₁, K⁺) did not record TD and [K⁺]o increased very slowly. Distant electrode (V₂) registered normal TD. Dashed line: control level of [K⁺]o.

Propagation and generation of the SD-related ΔV_o:

Neuronal transmembrane currents and syncytial or individual glial currents have all been considered as possible generators of the ΔV_o during SD in different brain nuclei (e.g., Nicholson and Kraig 1981; Tomita 1984). For SD of retina, Tomita (1984) has proposed that the typical ΔV_o are generated by glial Müller cells. Müller cells extend across much of the width of the retina, but the only cells in gray matter of hippocampus or neocortex showing a similar geometry that may support spatially arranged large extracellu-
lar sink-source distributions are pyramidal cells (e.g., Herreras 1990). A current sink occupying a large volume of tissue, surrounded by a weak but widely spread corona of current sources, could, however, be created in an extended system of electrically interconnected elements (Somjen 1973). Gial cells are believed to be more profusely connected by gap junctions than are neurons, so much so that gial cells are considered to form a functional syncytium (Kuffler and Nicholls 1967; Mugnaini 1986; Somjen 1973). Supporting the idea that SD depends on a network of widely spread electrically connected elements, agents that tend to close gap junctions, among them long chain alcohols, halothane, and acid pH, have all been shown to arrest the propagation of SD (Largo et al. 1997; Nedergaard et al. 1995; Saito et al. 1993). Over the years a number of investigators have proposed that neuroglia is the cell type primarily responsible for generating SD (Hertz 1965; Higashida et al. 1974; Sugaya et al. 1975). The discovery of so-called Ca$^{2+}$ waves that can spread among cultured astrocytes (Cornell-Bell et al. 1990) gave this idea new impetus, but except for the fact that both phenomena, Ca$^{2+}$ waves and SD, are slowly spreading events, there is no solid evidence so far to establish a causal relation. In fact, astroglial Ca$^{2+}$ waves may spread in more physiological preparations without indications of SD occurrence (e.g., Dani et al. 1992). Interestingly, Ca$^{2+}$ waves can also spread via gap junctions between cultured neurons and they do it at a faster speed (Charles and Tyndale 1995), more in agreement with that of SD.

There are other observations that favor neurons as the principal actors mediating SD generation and propagation. The blockade of the N-methyl-D-aspartate type of glutamate receptors, known to be present only in neurons, selectively blocks the main slow component of SD (Herreras and Somjen 1993a,b). Hull and Van Harreveld (1964) reported that SD failed to invade the territory of a glial scar, and inferred that glial tissue cannot support SD. Both neurons and gial cells depolarize during SD, but only neurons undergo a large increase of membrane conductance (Czéh et al. 1993; Somjen et al. 1993). Previously reported recordings (Herreras and Somjen 1993a,b) have shown that, before the onset of the major $\Delta V_m$, an oncoming wave of SD is heralded by a shower of “spontaneous” population spikes. Detailed analysis of this synchronous firing indicated that it can best be explained by electrotonic coupling among neurons (Herreras et al. 1994; see also Herreras and Somjen 1993a; Largo et al. 1996, 1997). Finally, the poisoning of glial metabolism and the consequent decrease of glial $V_m$ do not hinder SD propagation but rather favor it. Even while the glial $V_m$ decreased during FC treatment, the SD-related $\Delta V_m$ waves tended to expand. Only when neurons were impaired in their function did SD fail to invade an FC-treated area. This is also supported by the observation that while glial $V_m$ decreased to a similar extent in CA1 and neocortex during FC treatment, SD waves remained intact in the latter by the time they had already subsided in CA1, in close correspondence to the selective neuronal death in CA1.

**Mechanism of SD waveform changes during FC treatment**

The recovery of neurons from depolarization during SD requires active transport by neuronal membrane ion pumps to regain transmembrane ion gradients. Termination of SD is strongly dependent on energy supply (Leão 1947; Nicholson and Kraig 1981), and an overload of neuronal ion pumps could delay recovery and thus prolong the $\Delta V_m$ during FC treatment. The disappearance of the potassium undershoot is compatible with a gradual slow decay of energy availability, but this occurred much later than the changes in $\Delta V_m$, and therefore other more subtle mechanisms appear involved.

Given the relative independence of SD waves in stratum radiatum and pyramidale, we had previously suggested that there may be two different, although coupled, generators (Herreras and Somjen 1993a,b). The fact that the $\Delta V_m$ in the two strata became very nearly equal during FC treatment suggests, however, that the same generator could perhaps produce the slow component in both layers. If so, then variations in the density, distribution, assortment, or inactivation kinetics of ion channels could account for the different waveforms in the two strata. The equalization of SD waves by FC primarily acting on astrocytes should be then explained as a consequence of glial poisoning on neuronal function. Several changes are expected on glial metabolic derangement that will have strong modulatory effects on neuronal membranes, physiology, and metabolism, such as the extracellular pH or the size and composition of the extracellular matrix on one side, and intracellular pH, metabolites, or second messengers on the other (Kettenmann and Ramson 1995). However, as mentioned above, we cannot rule out a possible effect of FC directly on neurons.

In the present experiments, SD waves provoked in neocortex of untreated control preparations did not invade the CA1. SD passage among cortex and other regions has been reported (Bureš et al. 1992; Fifkova and Syka 1964; Vinogradova et al. 1991), but usually it is halted at the boundaries between nuclei. FC administration facilitated the passage of SD across such natural boundaries. The reason why FC-induced SD waves arose first in neocortex is not clear. That FC did spread to this region became apparent from a reexamination of histological specimens (Largo et al. 1996) that showed astrocyte and oligodendrocyte swelling also in the corpus callosum and lower cortical layers around the dialysis cannula. A very high susceptibility of cortical tissue to initiate SD during FC perfusion was noted, because SD was often triggered during simple readjustment of electrodes. It may be that neocortical astrocytes are more sensitive to FC than are hippocampal astrocytes (see Hassel et al. 1995).

**FC treatment, glial $V_m$, and potassium**

$V_m$ and input resistance of our sample of glial cells in control state were similar to those reported by Casullo and Krnjević (1986) in the same preparation. The gradual decrease of glial $V_m$ during FC administration is most probably caused by a shortage of metabolic energy and consequent loss of cytoplasmic K$^+$. The average decrease of glial $V_m$ reported here probably underestimates the true mean glial depolarization because of the deletion of the $V_m$ values less negative than $-30$ mV, as outlined in RESULTS. Nevertheless it is also clear that some glial cells maintained a relatively robust $V_m$ after many hours of FC treatment (Fig. 8). Even
though only glial cells are initially affected by FC, they do eventually recover after prolonged FC treatment, even after neurons have been irreversibly injured (Paulsen et al. 1987). Our data suggest that the depolarization is a function not only of dialysis time but also of the distance from the FC source, because depolarization was more severe in CA1 than in neocortex and fascia dentata. The wide variation of $V_m$ values during FC administration thus resulted from sampling cells that were scattered within a gradient of FC concentrations in the tissue. Alternatively, the closure of astrocytic gap junctions caused by the presumed internal acidosis (Anders 1988) may facilitate individual control of $V_m$ according to energy availability.

Assuming that the selectivity of the glial membrane for $K^+$ is preserved during FC treatment so that the glial $V_m$ remains to be a function of the ratio of $[K^+]_i/[K^+]_o$, inserting measured values for $V_m$ and $[K^+]_o$ in the Nernst equation, approximate values for $[K^+]_i$, can be calculated. Figure 9 illustrates the computed dependence of $[K^+]_i$ on the values of glial $V_m$ and $[K^+]_o$ within the range found for glial cells during FC treatment. A predicted loss of $K^+$ from glial cells is expected to be accompanied by a rise of $[K^+]_o$. In fact, however, the increase in $[K^+]_o$ during FC treatment in the absence of SD was moderate (Fig. 5). Part of the released $K^+$ ions may have been removed through capillary walls by circulating blood. Besides actual discharge of $K^+$ ions, dilution of the cytosol by cell swelling may have contributed in a small way to the lowering of $[K^+]_i$.

It is interesting to note that even after neurons are electrically inactive, slow negative $\Delta V_o$ still develop in parallel to extracellular potassium buildup (Largo et al. 1996; this report), at a time when SD can no longer be generated but some glia still preserve sizable $V_m$. In concordance with the above reasoning and earlier work by others (Dietzel et al. 1989; Gardner-Medwin 1983; Hertz 1965; Orkand et al. 1966; Roitbak et al. 1987; Somjen 1973, 1978) we may interpret this result as an indication that glia, even when partly deteriorated, cause the negative $\Delta V_o$ strictly related to $[K^+]_i$ buildup, whereas neurons are mostly responsible for the SD-associated $\Delta V_o$. It must be remembered that the $\Delta V_o$ during coalesced SD waves remain large, whereas the $[K^+]_o$ transients are reduced to a small elevated plateau (Herreras and Somjen 1993b), a result difficult to explain on the basis of spatial currents driven by $[K^+]_o$ gradients.

Is neuroglia a protector of neurons?

Our results indicate that normal glial function is an obstacle for SD spread. In our previous report (Largo et al. 1996) we suggested that the selective impairment of glial function imitates the condition of ischemic penumbra. Astrocyte swelling is the earliest reaction after several brain insults such as stroke (Kimelberg et al. 1990). Among the similarities was the generation of SD waves initiated near the dialysis probe but spreading into adjacent tissue. Recurrent SD waves appeared to be instrumental in the demise of neurons in FC poisoning, as they are in the ischemic penumbra (Hossmann 1994; Largo et al. 1996). It is known that astrocytes survive hypoxia much better than neurons. The neuronal injury caused by FC is most likely secondary to the loss of some supporting function by glial cells. We envisage glial tissue as limiting the duration of depolarization and constraining the spread of SD triggered by ischemia or other insults and therefore contributing to neuron survival in a variety of pathological conditions. On the other hand, faulty glial operation could increase neuronal risk by making the brain susceptible to long-lasting SD.

We thank Dr. G. Somjen for many helpful comments and suggestions. V. Abraira for assistance in the statistical analysis, and M.C. Botas for technical assistance.

This work was supported by grants from Fondo Investigaciones Sanitarias (FIS 93/661) and Dirección General Investigación Científica y Técnica (94/1257) of Spain. C. Largo is the recipient of a fellowship from the Spanish Ministry of Education (FP95).

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Received 19 December 1996; accepted in final form 27 March 1997.

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