Heptanol But Not Fluoroacetate Prevents the Propagation of Spreading Depression in Rat Hippocampal Slices

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INTRODUCTION

Spreading depression (SD) is among the most striking and reproducible electrophysiological phenomena in CNS gray matter, yet five decades after its discovery (Leão 1944) the mechanism by which waves of SD propagate is still not understood. For some time, two theories have dominated discussions of this topic: the potassium theory of Grafstein (1956) and the glutamate theory of Van Harreveld (1959).

There are, however, difficulties with both ideas. The facts that excess K⁺ can trigger SD and that K⁺ rises to very high levels during SD are well documented. If, however, the dispersion of K⁺ is the mechanism underlying the propagation of the SD wave, then at a point where brain tissue is about to be invaded by an advancing wave of SD, extracellular potassium concentration ([K⁺]o) should rise at first gradually up to a threshold level and then take off steeply. In fact, however, [K⁺]o remains steady up to the moment at which the advancing [V]o arrives; it then rises suddenly and steeply only slightly after the burst of population spikes and after the onset of the negative shift of extracellular potential (Herreras and Somjen 1993a; Lehmenkühler 1990). Although it is clear that, once [K⁺]o starts to rise it must influence the evolution of SD, apparently some process initiates SD even before diffusing K⁺ arrives, and that K⁺ ions cannot be the sole agent of SD propagation.

SD waves propagate even if axonal conduction and synaptic transmission are blocked completely (García Ramos and De la Cerda 1974; Herreras and Somjen 1993a; Tobiasz and Nicholson 1982). The absence of synaptic transmission does not, however, preclude the release of glutamate by means other than physiological transmission. If glutamate were the agent of SD propagation, then blockade of glutamate receptors should prevent it. Glutamate antagonists have indeed been found to increase the threshold of eliciting SD, slow down SD propagation, and limit the amplitude and duration of SD waves, but they do not completely abolish SD propagation at concentrations at which glutamate receptors are known to be blocked (Hernández-Cáceres et al. 1987; Herreras and Somjen 1993a; Marranes et al. 1988; Tegtmeyer 1993). To completely suppress SD, the dose of glutamate antagonists must be raised to levels where the specificity of the compounds was doubtful. These observations indicate that glutamate facilitates SD but is not essential for its onset or propagation.

Herreras and co-workers (Herreras and Somjen 1993a,b; Herreras et al. 1994; Somjen et al. 1992) proposed hypothetically that SD propagation is mediated not by the release of a substance from cells into interstitial fluid but by the intercellular transfer of a signal through the opening of interneuronal gap junctions that are normally closed. Among the observations that supported this idea was the eruption of a burst of population spikes of several millivolt amplitude ahead of an advancing [V]o, with spikes synchronized over distances of ≤1 mm (Herreras and Somjen 1993a,b; Herreras et al. 1994). Neuronal firing can be synchronized by ephaptic interaction of adjacent cells provided that interstitial resistance is high, as it is when cells are swollen (Traub et al. 1985). Cell swelling begins, however, at a given point...
at the same time as the shift of extracellular potential ($\Delta V_o$) (Jing et al. 1994) whereas the synchronized population spike shower usually precedes $\Delta V_o$ (Herreras and Somjen 1993a; Herreras et al. 1994). This early synchronization of firing over relatively long distances is best explained by assuming electrotonic continuity between neurons. It has been reported that electrical coupling among CA1 neurons is normally sparse (MacVicar and Dudek 1980; MacVicar et al. 1982) but Andrew et al. (1982) demonstrated dye transfer in 70% of injected CA1 pyramidal cells (see also Church and Baimbridge 1991; Knowles et al. 1982; Taylor and Dudek 1982). We suggested that ordinarily closed gap junctions open ahead of the advancing SD wave and, (as has been suggested earlier), also during seizures (Perez-Velasquez et al. 1994; Somjen et al. 1985).

Also supporting the role of gap junctions is the suppression of SD by acidois (Balestrino and Somjen 1988; Bureš et al. 1974) and by halothane, but not by chloralose (Saito et al. 1993). Halothane is known to block gap junctions (Mantz et al. 1993) but, besides this action, it is also a general anesthetic. Other support for a possible role of gap junctions came from a perceived similarity between the spread of calcium waves in cultures of astrocytes and that of SD in gray matter (Cornell-Bell et al. 1990; Finkbeiner 1992). Glial cells are believed to normally more profusely joined by gap junctions than are neurons. Whether or not glia is the tissue that conveys SD is, however, a matter of controversy (Czéh et al. 1992; Hull and Van Harreveld 1964; Largo et al. 1996a; Somjen et al. 1993; Sugaya et al. 1975).

To test the importance of intercellular communication in SD of mammalian brain tissue, we have assessed the effects of the long chain alcohols hexanol, heptanol, and octanol, which are known to block gap junctions (Johnston et al. 1980; Lee et al. 1994; Pott and Mechmann 1990), on SD propagation in hippocampal slices. To test the involvement of glia, we used the selective glial poison fluoroacetate (Clarke et al. 1970; Keyser and Pellmar 1994; Muir et al. 1986; Saito 1990; Szerb and Issekutz 1987).

While our study was underway, Nedergaard and collaborators (1995) and Martins-Ferreira and Ribeiro (1995) reported that both octanol and halothane block SD in isolated chick retina. Our observations in mammalian brain slices agree with their findings.

An abstract of some of these findings has been published (Largo et al. 1996b).

METHODS

Tissue preparation and drugs

Hippocampal slices were prepared from 80- to 150-g male Sprague-Dawley rats using standard techniques that have been described in detail elsewhere (Dingledine 1984; Somjen et al. 1986). Briefly, the ether-anesthetized animal was decapitated and the brain removed to ice-cold artificial cerebrospinal fluid (ACSF). One hippocampus was dissected free and cut into 0.40-mm-thick transverse slices on a MacIlwain type chopper. Slices were transferred immediately to cold ACSF and then divided between the two wells of a dual tissue slice chamber on a nylon mesh at the interface between warmed, humidified 95% O2-5% CO2 and oxygenated ACSF with the following composition (in mmol/l) 130 NaCl, 3.5 KCl, 1.2 CaCl2, 1.2 MgSO4, 1.25 NaH2PO4, 24 NaHCO3, and 10 glucose (pH 7.4, 34.5°C). ACSF flow rate was 1.5 ml/min. Slices were incubated for 90 min before beginning the experiment.

The 1 M stock solution of 1-octanol, 1-hexanol, or 1-heptanol (Sigma) in ethanol was diluted into ACSF with sonification immediately before use. Final concentrations were 3 mM (heptanol), 0.2–2.0 mM (octanol), and 5 mM (hexanol). Fluoroacetate (FAc)-containing ACSF was made by diluting a 1 M aqueous stock solution of the sodium salt of monofluoroacetic acid (Sigma) immediately before use to a final concentration of 5 or 10 mM.

SD triggering and recording

SD was triggered by means of a glass micropipette with its tip broken back to a diameter of ~5 μm, filled with 1.2 M KCl, connected to a Picospritzer (General Valve Corporation), and inserted in st. radiatum of the slice. Pressure pulses (1 or 2 pulses, 200–1,000 ms, 60 psi) reliably evoked SD in control slices. An Ag-AgCl wire sealed in the back of the KCl injection pipette and connected to a DC-coupled amplifier permitted recording both the DC potential ($V_o$) and the evoked potential at the injection site. $V_o$ was recorded at a second site ~1 mm distant with a 150 mM NaCl filled pipette. The injection/recording electrode is referred to as R1, the recording-only electrode as R2. The depth of each electrode was adjusted to obtain the maximal response evoked by orthodromic or antidromic potentials were recorded at R2 to permit that both octanol and halothane block SD in isolated chick retina. Our observations in mammalian brain slices agree with their findings.

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Dissociated cell whole cell recording

For patch-clamp experiments, pyramidal neurons were dissociated acutely from the hippocampal CA1 region of rat tissue slices according to the method of Kay and Wong (1986). Briefly, male Sprague-Dawley rats (100–125 g) were decapitated under ether anesthesia; 0.50-mm-thick hippocampal slices were prepared and small tissue pieces (~0.5 mm3) of the CA1 region were isolated and stirred gently for 90 min at room temperature in oxygenated N-2-hydroxyethylpiperazine-N‘-2-ethanesulfonic acid (HEPES)-buffered saline containing (in mM) 125 NaCl, 5 KCl, 1 CaCl2, 2
HEPTANOL AND SD

**FIG. 1.** A: diagram of experimental arrangement. R1, KCl injection pipette also used to record extracellular potentials; R2, extracellular recording electrode; S, stimulation electrode. For some experiments, a 2nd stimulation electrode was positioned in alveus for antidromic stimulation. B: effects of heptanol on spreading depression (SD) propagation. R1, extracellular potential at KCl injection site; R2, extracellular potential at distant recording electrode. Asterisks mark KCl injections: *200-ms pulse, * *400-ms pulse. Total elapsed time given at bottom. Calibration = 5 mV, 20 s.

**RESULTS**

**Effects of alkyl alcohols in brain tissue slices**

In six slices, SD propagation was completely blocked after 1–2 h of exposure to 3 mM heptanol and returned to control levels after washout of the heptanol (Fig. 1B). In one other slice, only partial blockade was observed and in another slice, SD was blocked but SD amplitude did not return to control levels during washout. In the presence of heptanol, the $\Delta V_o$ at the injection site became smaller, indicating decreased depolarization of the cells upon KCl injection. To be sure that true SD was elicited at the injection site during heptanol exposure, we routinely increased the size of the KCl injection until the local $\Delta V_o$ became comparable in amplitude with those obtained before heptanol application. With the local $\Delta V_o$ restored, propagated SD wave was occasionally detected, but its amplitude was blunted, and propagation eventually failed even with the increased KCl injections (Fig. 1B). Thus it appears that in addition to blocking SD propagation, heptanol also raised the threshold for evoking SD locally.

One might expect that SD propagation rate should gradually slow under the influence of a depressant drug before it completely fails. Surprisingly, as long as SD did occur in the presence of heptanol, its propagation velocity was not slower than that measured before or after heptanol administration. The mean rate of SD propagation fell gradually during the course of each experiment, but there was no significant difference during heptanol exposure compared with ethanol controls or with postheptanol recovery. The propagation rates were as follows (mm/min, mean ± SE, n = 5): control, 7.4 ± 0.6; EtOH, 6.3 ± 0.7; EtOH + heptanol, 5.6 ± 0.4; recovery in normal ACSF, 5.5 ± 0.5. These rates are similar
Heptanol significantly depressed evoked extracellular excitatory postsynaptic potentials (fEPSPs). Figure 2A shows individual fEPSP wave forms in heptanol, and Fig. 2B illustrates mean input/output curves. The initial fEPSP slope was reduced by an average of 30% at all stimulus intensities, an effect that reversed partially upon heptanol washout. Antidromic population spike amplitude was depressed by 30 ± 13%. At the concentration used (0.1–0.5%, 17–86 mM), ethanol (vehicle) did not significantly depress orthodromic or antidromic responses.

Other alcohols were tested for their ability to block SD. In five of six slices, octanol (1–2 mM) abolished SD propagation with partial (n = 2) or complete (n = 3) recovery upon washout. In the one remaining slice, SD propagation was unaffected by octanol. The source of this variability is not known, although it could reflect the relative insolubility of octanol in aqueous solutions as well as inadequate penetration into the tissue. Octanol concentrations >2 mM were not used. At concentrations of ≤0.5 mM, octanol had no detectable effect on SD (n = 2). In a separate series of experiments, hexanol (5 mM) also abolished SD propagation with full (3/6) or partial (3/6) recovery upon washout.

FIG. 2. Effects of heptanol on evoked potentials. A: extracellular excitatory postsynaptic potentials (fEPSPs) recorded before (cont.), during (hept.), and after (wash) application of heptanol. B: averaged input/output curves from 7 experiments. EtOH: 51.5 mM; heptanol: 3 mM.

Octanol and heptanol depressed the fEPSP to a similar extent (30 ± 5%, n = 6 for heptanol; 33 ± 14%, n = 3 for octanol); the depression caused by hexanol was more pronounced (65 ± 9%, n = 6). Overall, octanol was less consistent than either hexanol or heptanol in blocking SD, but the fact that all three alcohols had an effect strengthens our conclusion that gap junctions are required for SD propagation.

Effect of heptanol on voltage-dependent whole cell currents in dissociated neurons

The depression of antidromic spikes could have been caused by partial suppression of voltage-dependent Na⁺ currents. To determine whether heptanol affects Na⁺ channels, four dissociated CA1 pyramidal neurons were exposed to 3 mM heptanol. Na⁺ current peak amplitude was depressed by an average of 60% within 100 s of heptanol application. This effect reversed almost completely upon washout (Fig. 3).

Effects of fluoroacetate

In six of seven slices, exposure to 5 mM fluoroacetate (FAc) for periods ranging from 3 to 6 h failed to block SD propagation (Fig. 4). The ΔVₜ shift progressively increased in duration. Synaptic transmission was gradually suppressed over this period, as shown in Fig. 5, A and B. While the fEPSP began to decrease, there was sometimes a brief period of hyperexcitability during which multiple population spikes were observed in response to a single orthodromic stimulus. This hyperexcitability was not quantified, but its occurrence is consistent with the impairment of extracellular K⁺ buffering that would be expected with glial poisoning. Antidromic responses were not significantly affected by FAc (not shown).

Three additional slices were exposed first to 10 mM FAc and then to both FAc and 3 mM heptanol. SD propagation was unaffected by FAc alone but was blocked with the addition of heptanol, and recovered upon washout, with threshold raised in one case.

DISCUSSION

These observations clearly show the power of long chain alkyl alcohols to prevent the propagation of SD. In the presence of hexanol or heptanol, the local ΔVₜ at the site of the injection also was depressed, indicating diminished depolarization of the cells exposed to the injected KCl. The local depolarization could, however, be restored by raising the dose of injected KCl, yet SD propagation eventually failed even from this restored localized ΔVₜ (Fig. 1). It is also remarkable that, when SD did propagate under these conditions, its velocity was not slowed by heptanol, suggesting an all-or-none type conduction mechanism. That octanol had a similar if less reliably reversible effect reinforces the generality of this effect among alkyl alcohols. The concentrations of heptanol and octanol we used were higher than what is required to block gap junctions among cultured cells (e.g., Sontheimer et al. 1991) but were in the range used by others in brain tissue slices (Röorig et al. 1996; Yuste et al. 1995) and in retina (Martins-Ferreira and Ribeiro 1995; Nedergaard et al. 1995).
Heptanol also depressed both the evoked fEPSPs and the antidromic spike to a moderate degree. The depression of the antidromic spike probably is related to the partial suppression of voltage-dependent Na⁺ currents, as we observed in freshly isolated neurons, and has earlier been reported for cardiac muscle cells (Nelson and Makielski 1991) and for octanol in cultured astrocytes (Sontheimer et al. 1991). The mechanism of the synaptic depression is not clear but could be related to a depression of action potentials or of calcium currents in presynaptic terminals. However, neither failure of synaptic transmission nor block of Na⁺ channels can explain the failure of SD propagation caused by heptanol. In the presence of FAc, synaptic transmission failed, yet SD propagation was unimpaired. As has been repeatedly shown, blockade of voltage-gated Na⁺ channels by tetrodotoxin (TTX) also does not inhibit SD propagation (Garcia Ramos and De la Cerda 1974; Sugaya et al. 1975; Tobiaz and Nicholson 1982).

One property distinguishing heptanol from TTX, glutamate antagonists and other blockers of synaptic transmission is the ability to close gap junctions. The fact that heptanol succeeds in blocking SD propagation while TTX fails, strongly suggests that the opening of gap junctions is an essential step in the mechanism of propagation. There are other agents that can stop the spread of SD without necessarily preventing the localized SD-like depolarization generated at the site of the provoking insult. For example, nickel ions (2 mM) also blocked SD propagation,
Within suppressing the \( \Delta V_c \) at the site of high-\( K^+ \) application, or the SD-like depolarization caused by hypoxia (Jing et al. 1993). The mechanism of by which \( Ni^{2+} \) affects SD propagation remains to be explored. \( Ni^{2+} \) is known to block voltage-gated \( Ca^{2+} \) channels, but it may have other effects as well. Chebabo et al. (1993) reported the two local anesthetic agents, lidocaine and benzocaine arrest the circling SD in chick retina. To explain the difference between the SD block by local anesthetics and the lack thereof by TTX, Chebabo et al. (1993) point to the ability of the former to dissolve in the membrane lipid. Long-chain alkyl alcohols such as heptanol and octanol are also lipophilic. Whether or not local anesthetics interfere with gap junction function has, to our knowledge, not been explored. Earlier, Van Harreveld and Stamm (1953) reported that diethyl-ether does while pentobarbital does not block SD. Ether is another highly lipophilic compound. Whether it has an effect on gap junctions is, again, not known.

This leaves us with the conclusion that closure of gap junctions is the most likely but not the only possible explanation of the blockade of SD propagation by long-chain alkyl alcohols. If gap junctions are required for SD propagation, then the question arises whether interglial or neuronal junctions are involved. Numerous reports indicate that both fluorocitrate and FAc are selective poisons of glial metabolism (Berg-Johnsen et al. 1993; Clarke et al. 1970; Keyser and Pellmar 1994; Muir et al. 1986; Paulsen et al. 1987; Saito 1990; Szerb and Issekutz 1987). The lack of an effect on SD by FAc confirms earlier similar finding by Largo et al. (1996a) who administered fluorocitrate by microdialysis to hippocampus of intact brain of anesthetized rats. They reported that, similarly to FAc, fluorocitrate blocked synaptic transmission but did not interfere with SD. The anesthetized rats used by Largo et al. (1996a) were older than the ones from which we prepared our tissue slices, demonstrating that the findings were not peculiar to immature brain tissue. These observations suggest that intact glial function is not required for SD initiation or propagation. Largo et al. (1996a) also confirmed that 4 h of fluorocitrate microdialysis caused early morphological changes of glial cells while neurons were affected only after 8 h. It may be argued, however, that interglial gap junctions could continue to function even if the energy metabolism of the glial cells is impaired and, presumably, their membrane potential reduced or abolished.

Earlier observations also favor a major role for neurons rather than glial cells in the generation and propagation of SD waves. Hull and Van Harreveld (1964) found that SD failed to invade a cortical glial scar. In addition, voltage-clamp experiments in this laboratory revealed that during SD neurons exhibit a very large increase of input conductance, whereas glial cells show little or no change, even though the reversal potential (zero-current potential) of the glial \( I-V \) plot shifted in the depolarizing direction, as expected from the elevated \( [K^+]_o \) (Czéh et al. 1992; Somjen et al. 1993).

Taken together these diverse findings seem to suggest that SD propagation is mainly supported by neurons. Neuroglia may play a part in the process, but it does not seem to be the main mediator of its spread. We must then examine, whether interneuronal electrotonic junctions could support the SD process. Although gap junctions are abundant among glial cells (Mugnaini 1986), their density among neurons in adult nervous systems is less clear. There is increasing evidence that gap junctions play a role in the early development of gray matter, and the number of patent gap junctions appears to decrease with maturation of the brain (Kandler and Katz 1995). Both electrotonic and dye coupling among neurons, including pyramidal cells of CA1 region, however, has been reported in tissue slices as well as in intact brain of young as well as adult rats and guinea pigs (Andrew et al. 1982; Baimbridge et al. 1991; Knowles et al. 1982; Mac-Vicar et al. 1982) and such functional connections can be modulated by \( pH \) and by \( [Ca^{2+}]_o \), (Church and Baimbridge 1991; Perez-Velasquez et al. 1994). At least one report claims increase of electrotonic coupling in senescent rats (Barnes et al. 1987). In rats of 250–300 g body weight, gap junction protein associated with pyramidal neurons and granule cells has been located in all areas of the hippocampus, although less abundantly in the CA1 region than in CA2 and CA3 (Yamamoto et al. 1989).

Neither dye coupling nor the presence of immunohistochemically identified gap junction protein necessarily implies strong electrotonic coupling at rest (see e.g., Knowles et al. 1982). We have proposed, hypothetically, that SD propagation requires the opening of electrotonic junctions among neurons that may, under quiescent conditions, be

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**Fig. 5.** A: fEPSPs evoked during 5 h of fluoroacetate exposure showing gradual suppression of response. B: input/output curves from 5 slices showing changes in fEPSP slope caused by 5 mM fluoroacetate (2- and 5-h exposure).
closed. The data presented here are compatible with this hypothesis.

The preserved SD propagation in the face of blockade of synaptic transmission during FAc application confirms that synaptic transmission is not required for SD propagation. This does not preclude a nonsynaptic action of glutamate released from depolarizing neurons and glial cells. In our experiments, fEPSPs were depressed only moderately when heptanol blocked SD propagation. This indicates that SD propagation was blocked with glutamate receptors still functioning. Conversely, as already quoted in the INTRODUCTION, glutamate receptor blockade slows but does not prevent SD propagation (Hernández-Cáceres et al. 1987; Herreras and Somjen 1993a,b; Marranes et al. 1988; Tegtmeyer 1993). Therefore, it seems that glutamate facilitates but does not mediate SD propagation.

The suppression of synaptic transmission by these compounds, which has been reported previously (Keyser and Pellmar 1994; Largo et al. 1996a; Saito 1990), has been attributed to the failure of poisoned glial cells to supply glutamine, the precursor of glutamate, to neurons, or to swelling of glia that could disrupt synaptic structure (Largo et al. 1996a). Besides failure of synaptic transmission, the firing of multiple population spikes in response to single orthovoltage volleys suggests impaired glial function. Slowed clearing of excess [K+], could cause such hyperexcitability. The prolongation of SD waves during FAc treatment could have a similar explanation, except that SD waves tend to increase in duration when repeatedly provoked in anesthetized intact brains in the absence of FAc or other drugs (Chebabo et al. 1995; Herreras and Somjen 1993b). We did not examine whether the prolongation of SD-related ΔV, shifts under the influence of FAc is significantly greater than what would be seen otherwise.

The concentration of FAc required to block synaptic transmission (and, by inference, glial function) in rat hippocampal slices is much greater than that needed in the same tissue of guinea pigs (Pellmar and Keyser 1996). The higher dose used in our study could raise questions about the selectivity of the compound. However, the preservation of antidromic spike conduction in the presence of FAc demonstrates that neurons were viable, with well preserved membrane potential and membrane function.

Conclusions

There is much convergent evidence that gap junctions are required for SD propagation. Such junctions need not be open normally but must be capable of opening for SD to progress. Nevertheless, other explanations for the blocking effect by alkyl alcohols, for example the suppression of calcium currents, cannot be ruled out. Preserved SD propagation in the presence of the putative selective glial poison fluorocitrate on SD, taken together with other evidence in the literature, makes it unlikely that glial cells are essential for SD propagation.

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