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P/Q Ca^{2+} Channel Blockade Stops Spreading Depression and Related Pyramidal Neuronal Ca^{2+} Rise in Hippocampal Organ Culture

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Abstract

Ca^{2+} channels and pyramidal cell Ca^{2+} are involved in hippocampal spreading depression (SD), but their roles remain elusive. Accordingly, we characterized Ca^{2+} changes during SD in CA3 pyramidal neurons and determined whether Ca^{2+} channel antagonists could prevent SD. SD was induced in hippocampal organotypic cultures (HOTCs), in which experimental conditions can be rigorously controlled. SD was triggered by transient exposure to sodium acetate (NaAc)-based Ringer's coupled to an electrical pulse in the dentate gyrus and its occurrence confirmed with interstitial DC recordings. Pyramidal cell Ca^{2+} was measured with fura-2 filled cells and was quantified at the soma, proximal and more distal apical dendrites. Regional Ca^{2+} changes began simultaneously with the triggering pulse of SD and reached three distinct peaks before returning to baseline concomitant with the interstitial DC potential of SD. The first peak occurred within 5 s of the triggering pulse, was smallest, and heralded the onset of SD. The second Ca^{2+} change was the greatest and reached a peak 6 s later, during the early phase of SD. The third was intermediate in size and occurred 18 s later, as SD reached its maximum interstitial DC change. SD was prevented by nonselective Ca^{2+} blockade (Ni^{2+} and Cd^{2+}) but not by either L- Ca^{2+} channel (nifedipine) or N- Ca^{2+} channel inhibition (ω -conotoxin GVIA). Importantly, SD was blocked by P/Q Ca^{2+} channel antagonism (ω -agatoxin-IVA), which also prompted a significant reduction in pyramidal cell Ca^{2+} change and hyperexcitability. These results show that the spatiotemporal pattern of pyramidal cell Ca^{2+} change with SD is multiphasic; they provide further evidence that these changes begin before electrophysiologic evidence of SD. Furthermore, they show that P/Q Ca^{2+} channel antagonism can prevent SD in HOTCs and it appears to do so by preventing the NaAc-induced increased pyramidal cell excitability from NaAc exposure, which may involve altered GABAergic transmission.

Keywords

hippocampus; fura-2; GABA; inhibition; ion channels

INTRODUCTION

Ca^{2+} channels are fundamentally involved in Leão's spreading depression (SD) (Nicholson et al., 1977, 1978; Kraig and Nicholson, 1978), but their role in the initiation and/or propagation of this brain phenomenon remains elusive. It is known that Ca^{2+} entry via voltage-gated

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Ca²⁺ channels is essential for neurotransmitter release (Miller, 1987), and some investigators suggest that SD is triggered from a massive, simultaneous release of neurotransmitters (Somjen, 1973; Mori et al., 1976; Nicholson et al., 1981). Interstitial Ca²⁺ falls by more than 90% during SD (Nicholson et al., 1977), with at least some of this loss thought to enter presynaptic terminals and thus markedly enhance Ca²⁺-dependent transmitter release (Nicholson et al., 1978). In fact, non-selective Ca²⁺ channel antagonists can delay the initiation (Wauquier et al., 1985; Takagi et al., 1998) and block the propagation of SD (Jing et al., 1993). Also, generalized blockade of voltage-gated Ca²⁺ channels can retard the induction of SD in rat neocortex (Richter et al., 2002). Furthermore, adult transgenic mice with mutations in the $\alpha 1A$ subunit of P/Q Ca²⁺ channels have an impaired ability to undergo SD, perhaps due to reduced transmitter release (Ayata et al., 2000), although potential compensatory changes throughout ontogeny can confound interpretation of results from such animals (Gingrich and Roder, 1998). While these findings suggest a prominent role for Ca²⁺ in SD, they also indicate that the role of Ca²⁺ channels, and in particular P/Q-type Ca²⁺ channels, which are both pre- and postsynaptic (Usowicz et al., 1992; Westenbroek et al., 1995) are incompletely defined.

Ca²⁺ flux into neurons may be a key initiating event of SD. We previously observed two distinct Ca²⁺ waves preceding SD in hippocampal organotypic cultures (HOTCs) (Kunkler and Kraig, 1998). The first traveled rapidly along the pyramidal cell dendritic layer, while the second subsequently traveled more slowly and perpendicular to the cell layer. The first wave was suggested to reflect inter-pyramidal cell propagation via gap junctions. The second wave, which spread more slowly at a rate (i.e., mm/min) consistent with that of SD, but always ahead of it, might stem from inter-astrocyte propagation through gap junctions. Flux of Ca²⁺ into neurons, with the resulting decrease in interstitial Ca²⁺, might be the triggering event for the initiation of the slower inter-astrocytic Ca²⁺ waves (Kraig and Kunkler, 2002). However, the role, if any, of selective Ca²⁺ channels in the neuronal Ca²⁺ flux, which may initiate SD, has not been investigated in the hippocampus. Similarly, the spatiotemporal dynamics of neuronal Ca²⁺ change during SD have not been defined.

In this study, we show that SD in HOTCs occurs with three distinct pyramidal cell Ca²⁺ changes. The first is modest in size and heralds the onset of the interstitial DC negativity of SD. The second is the greatest and occurs early in SD, while the third is intermediate in size and slowest to reach a peak before returning to baseline with the interstitial DC potential of SD. Furthermore, nonspecific Ca²⁺ channel blockade could completely prevent SD while P/Q Ca²⁺ channel blockade also caused a nearly complete inhibition of SD. Importantly, P/Q channel blockade also significantly reduced the rise in neuronal Ca²⁺ and associated pyramidal cell hyperexcitability.

MATERIALS AND METHODS

Hippocampal Organotypic Culture Preparation

HOTCs are widely accepted experimental models (for review, see Bahr, 1995; Gähwiler et al., 1997) and were prepared and maintained as described previously (Stoppini et al., 1991; Kunkler and Kraig, 1997, 1998). Slices were maintained in vitro for 21–42 days prior to use, a period at least over which the cultures remain stable. For example, synaptic functional activity (Stoppini et al., 1991; Kunkler and Kraig, 1998; Schmitt et al., 2002) and susceptibility to SD are stable for at least 21–42 days in vitro. Furthermore, pyramidal cell viability (assessed by NeuN expression) and astrocytic reactivity (assessed by glial fibrillary acidic protein [GFAP] expression, a marker of tissue injury, degeneration, and inflammation) remain unchanged over this period in HOTCs (Schmitt et al., 2002). However, dendritic spine density of pyramidal cell apical dendrites in HOTCs (McKinney et al., 1999) resembles that seen in 15-day-old counterparts in vivo (Harris et al., 1992), perhaps due to reduced synaptic input. Nonetheless, AMPA- and NMDA-type glutamate receptors and other synaptic proteins in HOTCs remain

stable for at least 4 weeks in vitro (Bahr et al., 1995). Thus, since HOTCs display Ca^{2+} channel distributions similar to those found in vivo (Elliott et al., 1995), we presume that their expression remains stable over the period of our experiments. Indeed, the above findings and our experimental results discussed below support this contention.

Like acute brain slices, HOTCs are deafferented. However, the considerably longer survival of HOTCs allows sufficient time for some synaptic reorganization. For example, some CA3 and CA1 pyramidal cells synapse back onto CA3 pyramidal neurons (Debanne et al., 1995). CA1-CA1 synapses and synapses from CA3 to dentate granule cells are also seen (Gutierrez and Heinemann, 1999). Nonetheless, these aberrant connections do not appear to be a dominant confounding factor for the use of HOTCs. This conclusion follows from the fact that the basic trisynaptic loop (i.e., DG-CA3-CA1) is preserved both structurally (Zimmer and Gähwiler, 1984) and functionally (Gutierrez and Heinemann, 1999) in HOTCs. Furthermore, HOTCs show CA1 and CA3 selective neurotoxicity to injurious insults like that seen in vivo (Kristensen et al., 2001). Finally, HOTCs exhibit intrinsic patterns of GABAergic transmission similar to that seen in vivo (Streit et al., 1989).

Electrophysiologic Recording

For electrophysiologic recordings, HOTCs on a Millipore insert were placed in a 35-mm culture dish, mounted in a movable open perfusion micro-incubator (PDMI-2; Medical Systems; Greenvale, NY) on an inverted microscope (DM IRBE; Leica Mikroskopie und Systeme GmbH; Wetzlar, Germany) sitting on a specially designed Gibraltar frame (Burleigh Instruments) as previously described (Kunkler and Kraig, 1998). HOTCs were perfused with a normal Ringer's solution containing (in mM): NaCl 124, KCl 2, NaHCO_3 26, CaCl_2 2.5, MgCl_2 1, KH_2PO_4 1.2, glucose 6 (290–300 mOsm), adjusted to pH 7.3–7.4 with 5% $\text{CO}_2/95\%$ O_2 and maintained at 36°C. The perfusate was directed within the insert for the first few minutes after it was mounted into the recording chamber. Then, the Ringer's solution was directed outside (i.e., around and beneath) the insert at a rate of 1–2 ml/min. Next, the recording solution was gently “wicked” off the surface of the insert membrane using cotton-tipped applicators and HOTCs were covered with light mineral oil to prevent dehydration.

To record evoked responses, an interstitial microelectrode (tip diameter 4–6 μm) filled with 150 mM NaCl was driven into the culture with a piezoelectric micromanipulator (PCS-5000; Burleigh Instruments). A bipolar (90% platinum/10% iridium) twisted Teflon insulated wire (125 μm diameter; 7780, A-M Systems; Everett, WA) stimulating electrode was placed gently on top of the dentate gyrus (DG). A 1 M KCl agar bridge ground electrode was placed outside of the insert and within the 35-mm culture dish beneath the level of the perfusate. Stimulating pulses were 100 μs in duration and 20–50 V in intensity at a constant current setting, using a stimulator (World Precision Instruments 1800 series) and an associated stimulus isolator. Interstitial DC signals were monitored using an A-1 Axoprobe amplifier system (Axon Instruments, Foster City, CA), digitized with a 1200 series Digidata system (Axon Instruments), and processed using Axoscope software (version 8.0; Axon Instruments). Separate Pentium AST computers (AST Research, Irvine, CA) were used to acquire fast and slow signals. Fast evoked signals were sampled every 100 μs ; slow potential recordings were sampled every 0.1–5 ms.

To initiate SD, the perfusate was switched to a modified Ringer's solution in which NaCl was replaced with an mM equivalent of sodium acetate (NaAc) (pH 7.60) (Bureš et al., 1974; Kunkler and Kraig, 1998). This modified Ringer's was pulsed on for 2 min, and a SD episode initiated with a single pulse from the bipolar stimulating electrode. Following return of the interstitial DC signal toward baseline levels (<1 min), perfusate was switched to normal Ringer's. To ensure SD-initiation reproducibility, SD was initiated again after a 10-min recovery period.

Fluorescence Imaging

For intracellular Ca^{2+} imaging, CA3 pyramidal neurons were impaled with microelectrodes pulled on a Brown-Flaming puller (model P-97; Sutter Instrument, Novato, CA) from 1.5-mm borosilicate glass (A-M Systems, Everett, WA). The microelectrodes were filled with a solution containing 10 mM fura-2 (Molecular Probes, Eugene OR) in 0.5 M KCl or 0.5 M potassium acetate and had a final resistance of 80–120 M Ω . After cell impalement, neurons were injected with iontophoretic current (400-ms pulses at 2 Hz; -1.0 to -1.5 nA) for 10–20 min. The Ca^{2+} indicator fura-2 was used due to volume changes known to occur during SD (Hansen and Olsen, 1980; Aitken et al. 1999), thus making the use of a ratiometric indicator necessary. Cells having a stable resting potential more negative than -50 mV after dye injection were used in experimental manipulations.

For whole-culture voltage imaging, HOTCs were stained for 1 h with the voltage-sensitive dye RH795 (Molecular Probes) while being gently rocked on a rocker platform at room temperature. The dye (7 $\mu\text{g}/\text{ml}$), dissolved in a Hepes buffered salt solution (HBSS) containing 0.1% dimethylsulfoxide (DMSO), was placed on top (0.8 ml) and under (1 ml) the insert to enhance staining. The cultures then were washed for an additional 30 min in HBSS before transfer to recording chamber.

Fluorescence was elicited by excitation with a TILL Photonics Polychrome II monochromator (Applied Scientific Instruments, Eugene, OR) using a 75-W xenon short arc lamp. Images were acquired using a 12-bit Pentamax CCD camera with a GEN IV intensifier (Roper Scientific) and Metafluor Imaging system software (version 4.64; Universal Imaging, West Chester, PA) run via a Pentium-500 MHz computer (Micron Electronics, Boise, ID). Fura-2 images (256×256 pixels; 1×1 binning) were acquired using 340/380-nm excitation at 1.5 Hz (200–100 ms per image, respectively) and a $\times 20$ objective. All fura-2 images were corrected for background fluorescence using images acquired from adjacent HOTCs. Voltage dye imaging (512×512 pixels; 2×2 binning) experiments were acquired at 4 Hz (100-ms exposure per image) with a $\times 6.3$ objective, using 530-nm excitation. Temporal synchrony of the simultaneous electrophysiologic and imaging measurements was ensured by recording a TTL voltage pulse from the camera trigger along with all slow DC signals from HOTCs as noted above.

Tissue autofluorescence from NADH conceivably might confound fura-2-based measurements. This conclusion stems from the fact that NADH fluoresces in the blue region upon excitation with near-ultraviolet light (e.g., 365 nm) (Puppels et al., 1999), a wavelength of light near the 380 and 340 nm light used for fura-2 excitation. Furthermore, others have noted either a decrease (Sonn and Mayevsky, 2000) or an increase (Hashimoto et al., 2000) in NADH fluorescence from neocortical SD in vivo. Accordingly, we measured the relative contribution of tissue autofluorescence (i.e., approximate change in NADH fluorescence; $n = 9$) during SD by registering a separate (but similarly sized) area of interest away from fura-2 cells but within the HOTC tissue and comparing this to fura-2 somatic changes elicited by 380- and 340-nm light excitation. Average change in fluorescence intensity versus these two sites and at these two wavelengths during SD was 19 ± 2 vs 1538 ± 86 and 23 ± 4 vs 647 ± 118 intensity units (IU), respectively. Thus, SD-related changes in autofluorescence amount to 1% and 4% of the fura-2 changes at 380- and 340-nm excitation, levels that would not significantly influence measured fura-2, and therefore pyramidal cell Ca^{2+} changes. Kovacs and coworkers (2001) recently showed similar low percentage changes in NADH fluorescence during seizures in HOTCs.

Pharmacological Agents

Stock solutions of the Ca^{2+} channel antagonists with the exception of nifedipine were dissolved in distilled H_2O . Nifedipine (Sigma) was first dissolved in 95% ethanol before being added to

Ringer's solution, resulting in a final ethanol concentration of $\leq 0.05\%$. Preliminary studies revealed no effect of this ethanol concentration on evoked field responses or SD induction. Nifedipine was protected from ambient light following the manufacturer's recommendations. Stock solutions of the calcium antagonist ω -conotoxin GVIA (Ctx-GVIA) and ω -agatoxin-IVA (AgTX) (Bachem) were frozen in single-use aliquots at -20°C . Cytochrome c (Sigma; 0.1 mg/ml) was combined with solutions containing Ctx-GVIA and AgTX to saturate nonspecific peptide binding sites (Wheeler et al., 1994). In control experiments, cytochrome c was shown to have no effect on the SD initiation ($n = 5$), but did appear to decrease the fura-2 signal intensity. Reducing the concentration (0.02 mg/ml) during fura-2 imaging eliminated this effect with no apparent effect on efficacy of the antagonist. Stock solutions of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma) were dissolved in 0.1 N NaOH. DL-2-aminophosphonovaleric acid (APV; Fluka), NiCl_2 , and CdCl_2 were dissolved in H_2O . KH_2PO_4 was omitted from Ringer's solution in experiments using Ni^{2+} or Cd^{2+} to prevent precipitation.

All data are presented as mean $\pm$ SEM values with statistics performed and results obtained as indicated using Sigma StatTM software (version 2.03; Chicago, IL).

RESULTS

SD Initiation in HOTCs

In normal Ringer's, electrical stimulation of the DG induced reproducible evoked field potentials in the pyramidal layer of CA3 (Fig 1A). Electrical stimulation induced field potentials consisting of a large rapid population spike followed by an equally large field excitatory postsynaptic potential (fEPSP) lasting 10–20 ms. Repetitive (0.1–1.0 Hz) stimulation induced field potentials that did not deteriorate nor initiate more than a single response per stimulus. However, by briefly switching from normal Ringer's to NaAc Ringer's, SD was reliably induced in HOTCs (Kunkler and Kraig, 1998). This modified Ringer's provided a sufficient "conditioning" change to induce SD by a single bipolar electrical stimulus to the DG. Figure 1B shows the typical interstitial DC changes of SD in HOTCs, namely a large, transient and principally negative shift in DC potential that is associated with a transient loss in evoked field potentials, changes similar to those originally defined *in vivo* (Leão, 1944; Bureš et al., 1974).

The interstitial DC signal of SD in HOTCs consisted of a very rapid deflection followed by a characteristic "inverted saddle" waveform similar to that observed in the *in vivo* hippocampus (Herreras and Somjen, 1993). Peak negative interstitial DC potentials of 30–50 mV were often observed, followed by a DC shift toward baseline with occasional spontaneous epileptiform activity present, a phenomenon previously noted to occur in hippocampal brain slices derived from adult (Snow et al., 1983) and immature (Psarropoulou and Avoli, 1993) rats. Throughout the negative DC shift, evoked electrical activity was absent but, during the recovery phase of SD, evoked field potentials gradually recovered, first only as exaggerated fEPSP, then progressing to include population spikes (Fig. 1B).

The effects of tissue "conditioning" by NaAc Ringer's were also reflected in the triggering field potential. Compared to field potentials evoked in normal Ringer's, several unique changes in the waveform after conditioning were noted that were consistent with enhanced excitability. The first change was an enhanced and wider population spike. The second consisted of multiple population spikes present during the fEPSP. The third and most pronounced change was the presence of a prolonged fEPSP, the duration of which lasted 100–200 ms (e.g., compare first, darkened for visualization, evoked field potential in Fig. 1A,B).

To test whether reduced pyramidal cell inhibition is important for triggering SD, we examined the effect of the GABA_A antagonist, bicuculline (Sigma) on SD. Bicuculline (20 μ M and 100 μ M) increased HOTC spontaneous excitability and triggered spontaneous and recurrent SDs within minutes of exposure that continued for 1–2 h. Then, the synchrony of such spontaneous, recurrent SDs degraded to irregular, recurrent, and spontaneous seizures ($n = 5$; data not shown). The latter results are consistent with those seen with long-term (e.g., >2 min) exposure to NaAc-Ringer's. Similarly, exposure to gabazine (SR-95531; Sigma) (200 nM or 10 μ M) produced only seizures, and not SD.

The third classical electrophysiologic change associated with SD is a slow propagation of a large negative interstitial DC potential (Leão, 1944). To illustrate that this third defining criteria of SD also occurs in HOTCs, sections were stained with the fluorescent voltage-sensitive dye RH795 prior to SD initiation. RH795, which decreases in fluorescence intensity with cellular depolarization, allows for the detection of cellular depolarization and SD propagation throughout the HOTCs. Two microelectrodes were also placed into CA3 and CA1 to further confirm SD propagation. Figure 2 shows an HOTC stained with RH795 and imaged during SD with concomitant interstitial DC potential traces from CA3 and CA1. Following perfusion in NaAc Ringer's, a single field stimulus induced a rapid -10 -mV drop in the CA3 DC potential, initiating SD. This drop in potential is associated with a drop in CA3 fluorescence, which slowly propagates toward CA1 over the next 30 s, and is also evident on the CA1 interstitial DC potential trace. Propagation velocity, as measured by tissue fluorescence changes (2.63 mm/min ± 0.61 ; $n = 7$), is consistent with previous reports in HOTCs (Kunkler and Kraig, 1998) and in vivo (Leão, 1944; Bureš et al., 1974). Thus the three defining hallmarks of SD also are found in HOTCs, further demonstrating this in vitro CNS tissue model has characteristics similar to those found in vivo (Kraig and Kunkler, 2002).

Neuronal Ca²⁺ Transients During SD

To explore whether the known decrease in interstitial Ca²⁺ during SD occurs specifically with a neuronal Ca²⁺ rise, we imaged CA3 pyramidal neurons filled with fura-2 during SD (Fig. 3). Fura-2 was used as a qualitative and spatiotemporal measure of neuronal Ca²⁺ behavior and quantitated in three distinct zones (soma, proximal apical dendrites (i.e., 50–75 μ m from the cell soma) and more distal apical dendrites (i.e., 125–150 μ m from the soma)) (Fig. 3A). Baseline fura-2 fluorescence ratios were not significantly different ($P=0.972$; analysis of variance [ANOVA]) at these three zones, and their collective mean baseline fluorescence ratio level was 0.7 ± 0.1 IU ($n = 6$ cells and 18 recording zones). A single field stimulus induced a prompt rise in neuronal Ca²⁺ and triggered SD only if accompanied by exposure to NaAc Ringer's (also, see below). The rise followed a stereotypic pattern consisting of three distinct peaks (that was most evident in the dendrites) before returning to baseline with the interstitial DC potential change of SD (Fig. 3B,D). The first peak occurred variably, but within 5 s of the triggering pulse, and reached a peak fluorescence change of 6.2 ± 1.1 , 3.7 ± 0.8 , and 1.5 ± 0.6 IU in the more distal dendrites, proximal dendrites and soma, respectively, which heralded the onset of SD. The second peak occurred 6.1 ± 0.8 s after the triggering pulse, and with the development of the saddle-shaped interstitial DC change of SD, and reached peak fluorescence changes of 14.9 ± 3.2 , 6.6 ± 1.7 , and 3.4 ± 1.1 IU at these same zones, respectively. The third peak occurred 17.6 ± 0.9 s after the second peak and as the interstitial DC change of SD reached its maximum with peak fluorescence changes of 5.3 ± 1.5 , 2.4 ± 0.5 , and 2.1 ± 0.5 IU, respectively. Correlations between pyramidal cell Ca²⁺ changes (Fig. 3B) and interstitial DC changes (Fig. 3D) were based on our prior observation that tissue Ca²⁺ changes from SD in HOTCs begin within 1–2 s of the initial DC deflection (Kunkler and Kraig, 1998). Here, we presumed the first Ca²⁺ peak seen in pyramidal neurons corresponds to the initial tissue Ca²⁺ change previously noted during SD.

Regional peak fura-2 fluorescence changes were summed and converted to percentage changes to enhance comparison of changes between cells. The second peak Ca^{2+} rise of SD was significantly greater ($P < 0.001$; ANOVA with post-hoc Tukey) than the other two peaks in the dendrites. The magnitude of the second peak was less dramatic in the soma. There the second peak remained significantly greater ($P < 0.05$; ANOVA with post-hoc Tukey) than the first peak, but not the third. This is evident by the comparatively dampened rise and fall of the Ca^{2+} change in the soma (Fig. 3B).

To provide a perspective of the neuronal Ca^{2+} changes from SD, these results were compared to those seen after evoked field potentials. Under our recording conditions, a Ca^{2+} change from a single DG evoked field potential was not detected (Fig. 4). However, CA3 pyramidal cell fura-2 ratios rose from a baseline of 1.0 ± 0.1 ($n = 6$ cells) to show a peak change after 10–20 s of 5-Hz repetitive DG stimulation of 1.8 ± 0.4 at the soma (data not shown). This is comparable to the change seen after a single, DG evoked stimulus (i.e., first, somatic peak noted at $> 1.5 \pm 0.6$) during NaAc exposure associated with triggering SD. However, in our experience, 5-Hz stimulation alone is not capable of eliciting SD.

Ca^{2+} Channel Blockers and SD

In HOTCs where SD was successfully induced on two successive trials, Ringer's solution was subsequently switched to one containing either Ni^{2+} or Cd^{2+} to determine whether blocking calcium channels would inhibit SD initiation. Initial concentrations of these divalent ions were based on previously reported attempts to block SD in hippocampal acute slices (Jing et al., 1993). The HOTCs were exposed to the modified Ringer's containing Ni^{2+} or Cd^{2+} for 30 min, after which time the ability to evoke field potentials and SD was assessed. In Ringer's containing either 1 mM Ni^{2+} or 50 μM Cd^{2+} , evoked field potentials were similar in amplitude to those obtained in normal Ringer's, but the fEPSP duration was slightly diminished (data not shown). Although evoked field potentials were obtained, fields evoked after a 2-min perfusion in NaAc Ringer's containing Ni^{2+} produced a 100% block of SD ($n = 5$). Extending the perfusion time in NaAc Ringer's to 10 min still produced a 100% block of SD ($n = 5$) while lowering Ni^{2+} to 500 μM , partially reversed this effect so that only 22% of trials blocked SD ($n = 9$). Cd^{2+} (50 μM) likewise completely blocked SD ($n = 9$). Increasing the Ni^{2+} concentration to 5 mM completely eliminated DG evoked field potentials in CA3 ($n = 5$).

Inhibition of SD by Ni^{2+} or Cd^{2+} suggests that Ca^{2+} flux across cell membranes may play a role in triggering SD in HOTCs. However, since these divalent ions affect other channels besides voltage-gated Ca^{2+} channels (Hille, 1968; Ault et al., 1980), we examined whether specific Ca^{2+} channel antagonists could block SD initiation in HOTCs (Table 1). Consistent with in vivo results (McLachlan, 1992), perfusion of the NMDA receptor antagonist APV (50 μM) produced an 83% block (e.g., 5 of 6 trials) of SD initiation. In contrast, the selective non-NMDA receptor antagonist CNQX (20 μM), did not block SD initiation ($n = 6$), but did block evoked field potentials (and thus SD because of the absence of needed DG-evoked field potential in CA3) at 100 μM . Likewise, the L-channel antagonist, nifedipine (10 μM) and the N-channel blocker Ctx-GVIA (2 μM) had no effect on triggering SD ($n = 6$). However, the P/Q-channel blocker AgTX blocked SD initiation in a concentration dependent manner. For example, low concentrations (20 nM) had no effect but higher concentrations (175 nM) produced a 92% block of SD initiation ($n = 13$).

The divalent ions Ni^{2+} and Cd^{2+} have been reported to block SD propagation but not its initiation in acute hippocampal brain slices (Jing et al., 1993). To determine whether similar phenomena occurred in HOTCs, we repeated our divalent ion experiments while monitoring for SD propagation, using the voltage dye RH795 (data not shown). Consistent with the results noted above using single microelectrode recordings, no evidence of either initiation or propagation was observed in tissue perfused with either 1 mM Ni^{2+} ($n = 4$) or 50 μM Cd^{2+} (n

= 5). Similar results were observed with 175 nM AgTX (n = 4). In all experiments, no change in interstitial DC signal was evident, thus confirming the blockade of SD in HOTCs.

AgTX Inhibition of SD

To address the effects of AgTX on SD and its initiation more directly, fura-2 was injected into CA3 pyramidal neurons and imaged during SD initiation (Fig. 4). In normal Ringer's, a single evoked field potential induced no detectable change in the neuronal Ca^{2+} level under our recording conditions. Application of 175 nM AgTX for 30 min completely blocked SD initiation (n = 3) and reduced the initial neuronal Ca^{2+} rise from the triggering pulse. Neuronal Ca^{2+} rose in conjunction with the electrical stimulus but far less so (0.3 ± 0.1 IU change for the first Ca^{2+} peak compared to 1.5 ± 0.6 , as shown in Fig. 3, and 1.8 ± 0.4 from 5-Hz repetitive DG stimulation under normal Ringer's exposure). Continued application of AgTX further reduced the neuronal Ca^{2+} response.

AgTX exposure also altered the characteristics of field potentials evoked during exposure to NaAc Ringer's. AgTX caused little effect to evoked field potentials under normal conditions (data not shown), but this was not the case for conditions used to trigger SD (Fig. 5). For example, time to peak fEPSP from the stimulus artifact was 10 ± 1 ms in normal Ringer's (n = 5). In contrast, NaAc Ringer's led to a significant increase ($P < 0.001$; ANOVA with post-hoc Tukey) in time to peak fEPSP (42 ± 4 ms; n = 5) compared with evoked field potentials acquired in normal Ringer's. AgTX exposure however, reversed this NaAc-induced significant increase in fEPSP back to a nonsignificant difference in time to fEPSP peak in normal Ringer's (9.4 ± 1 ms; n = 5).

DISCUSSION

This work includes several novel findings that may be important in further deciphering the mechanisms responsible for SD (Kraig and Kunkler, 2002). First, we have shown that SD in HOTCs is associated with three distinct pyramidal cell Ca^{2+} changes: one that occurs with the triggering pulse, and two that occur during SD. Second, these latter Ca^{2+} changes and SD in HOTCs were prevented by specific antagonism of a single ion channel type, namely the P/Q Ca^{2+} channel by AgTX, while the first Ca^{2+} peak associated with triggering SD was diminished. Third, P/Q Ca^{2+} channel blockade reduced to normal the NaAc-induced increase in pyramidal cell excitation, which may be necessary for SD induction in HOTCs.

Pyramidal Cell Ca^{2+} Changes and SD

Recognition of the potential importance of Ca^{2+} in SD perhaps began in earnest with the classical experiment of Nicholson et al. (1977). These workers showed for the first time that interstitial Ca^{2+} fell more than 90% during SD. Logically, most immediately assumed the loss of Ca^{2+} from the interstitial space occurred because of an associated transmembrane flux into neural cells (Kraig and Nicholson, 1978; Siesjö and Bengtsson, 1989). However, no direct evidence existed to support his contention until decades later, when Basarsky et al. (1998) and Kunkler and Kraig (1998) used fluorescent markers for cellular Ca^{2+} to show that intracellular Ca^{2+} levels began to rise seconds before the classical electrophysiologic concomitants of SD (Bureš et al., 1974) in acute, hippocampal brain slices and HOTCs, respectively. Earlier work from this lab provided evidence, using ion-selective microelectrodes, showed that astrocytic Ca^{2+} rises during the electrophysiologic changes of SD (Kraig et al., 1995). However, until now, neuronal Ca^{2+} behavior during SD has not been defined.

Our measurements in this study show several fundamental features of pyramidal cell Ca^{2+} change during SD. First, Ca^{2+} changes rise faster and reach higher peak levels in dendrites than in the soma, a pattern of change noted previously for pyramidal cell Ca^{2+} changes from

evoked electrophysiologic activity (Pozzo-Miller et al., 1993). Such differentially centripetal Ca^{2+} changes are reminiscent of similar spatiotemporal activity-dependent changes seen in neurons (Llinás and Hess, 1976; Schwartzkroin and Slawsky, 1977; Wong et al., 1979) suggesting they may trigger changes in cellular function (Llinás, 1988; Berridge, 1998) common to both normal physiology and SD. Second, the pyramidal cell Ca^{2+} change from SD is triphasic. An initial rise occurred before the interstitial DC component of SD. This adds to the evidence that cellular Ca^{2+} changes of SD precede the biophysical changes of SD (Basarsky et al., 1998; Kunkler and Kraig, 1998). Furthermore, it adds credence to the notion that such antecedent events include pyramidal neurons (Kunkler and Kraig, 1998). Perhaps, this Ca^{2+} change is a trigger needed for SD. Alternatively, it may simply be a correlate of triggering SD made evident by a sufficiently enlarged volume of synchronously depolarized tissue (Bureš, et al., 1974) from the electrical stimulus during NaAc exposure. The second rise in pyramidal cell Ca^{2+} was always greatest and occurred with development of the interstitial negativity of SD. This is a time when neural cell membrane permeability is maximal during SD (Kraig, 1990), suggesting that the Ca^{2+} rise here especially was due to flux from the interstitial space. The source for the third rise in Ca^{2+} is less certain though its delayed time course suggests involvement of intracellular stores (Pozzo-Miller et al., 2000; Rose and Konnerth, 2001).

Fura-2 ratiometric measurements of pyramidal cell Ca^{2+} change never saturated during SD, indicating Ca^{2+} changes from SD occurred within the sensitivity range of this indicator dye. Fura-2 accurately measures cellular Ca^{2+} change from 0.1 to 10 times the K_D (Haugland, 2002), and the fura-2 K_D in situ is reported to be 371 ± 71 nM (Petr and Wurster, 1997). Thus, the Ca^{2+} changes reported here for the SD were probably within 0.4 nM to 4 μM . For example, we could not detect a CA3 pyramidal cell Ca^{2+} change to a single evoked stimulus. However, we could detect a change to 5-Hz repetitive DG stimulation. In fact, the fura-2 ratio change to 5-Hz stimulation (1.8 ± 0.4) was similar to that seen with the triggering pulse for SD (i.e., the first somatic Ca^{2+} peak of 1.5 ± 0.6). This is comparable to the fura-2 change that occurs in CA1 pyramidal neurons activated by depolarizing pulses (Smetters et al., 1999). The greatest fura-2 ratio change occurred early in the electrophysiologic change of SD and in the distal dendrites, where a ratio increase of 14.9 ± 0.3 was seen. This may correspond to an approximate concentration peak of less than a micromole (Haugland, 2002), since baseline Ca^{2+} is ~ 30 nM in CA3 pyramidal neurons within HOTCs (Knöpfel and Gähwiler, 1992). Interstitial Ca^{2+} falls from ~ 1.2 mM to <0.1 mM during SD (Nicholson et al., 1977), and interstitial space is normally about 20% of brain volume (Nicholson and Phillips, 1981). If this fall in interstitial Ca^{2+} occurred with free distribution into the intracellular space, Ca^{2+} level within cells would be expected to rise to ~ 240 μM . Since they do not, cellular Ca^{2+} buffering must occur during SD, which may also influence triggering events related to SD as well as its subsequent consequences to involved brain tissue (Berridge, 1998; Rose and Konnerth, 2001).

P/Q Ca^{2+} Channel Blockade

Nonspecific Ca^{2+} channel antagonism previously has been shown to effect the initiation and propagation of SD in acute brain slices (Wauquier et al., 1985; Jing et al., 1993; Footitt and Newberry, 1998; Takagi et al., 1998) and in vivo (Marrannes et al., 1993). However, deciphering whether such changes are due to Ca^{2+} channel function per se is made difficult by other nonspecific effects of the antagonists. For example, Takagi et al. (1998) observed that the nonselective Ca^{2+} channel antagonists flunarizine and lomerizine prolonged both the latency and interval between SD in acute slices. In addition, Marrannes et al. (1993) found that flunarizine reduced the frequency and duration of SD in vivo. However, whether this is due to specific changes in tissue Ca^{2+} homeostasis remains uncertain because these agents also affect other ion channels (Pauwels et al., 1986), which may be important to triggering and sustaining SD (Müller and Somjen, 1998). Similarly, Jing et al. (1993) used Ni^{2+} , a nonselective Ca^{2+} channel antagonist, to impair SD induction in acute hippocampal slices. However, they only

achieved 50% inhibition at the extreme concentration of 2 mM, a level likely to induce effects on other ion channels (Hille, 1968; Ault et al., 1980). In fact, when used at low dose (i.e., 10 μ M), prolonged exposure to Ni^{2+} enhances susceptibility to SD in acute hippocampal slices (Gorji et al., 2000) perhaps due to electrophysiologic effects caused by relatively specific blockade of T-type Ca^{2+} channels (Mogul and Fox, 1991; Beck et al., 1997; Tang et al., 1988).

To explore further whether Ca^{2+} channel blockade could stop SD, we turned to specific antagonists and showed that P/Q-type Ca^{2+} blockade stopped SD in 92% of trials (12 of 13). This result supports and extends the observations made by Ayata et al. (2000) who showed that mice with mutations in the $\alpha 1A$ subunit of the P/Q type Ca^{2+} channel show an elevated threshold for neocortical SD. However, as noted above, results with such genetically altered animals can be confounded by potential compensatory changes throughout ontogeny (Gingrich and Roder, 1998). Indeed, Richter and coworkers (2002) recently showed that SD could be retarded in neocortex of anesthetized rats by either N-, L-, or P/Q-type Ca^{2+} channel antagonism, when applied locally. In contrast, the L- and N-specific channel antagonists nifedipine and ω -conotoxin GVIA had no effect on SD in the HOTCs. Perhaps such differences in the effects of Ca^{2+} channel modulation on susceptibility to SD are due to regional differences (i.e., between neocortex and hippocampus), differences in channel expression among animal models (i.e., acute slices versus HOTCs) or methods of inducing SD (i.e., hypoxia exposure in acute slices (Somjen, 2001) versus KCl or anion substitution plus electrical stimuli in HOTCs). If so, then our results here may be comparable to hippocampal SD in vivo since Ca^{2+} channel distribution in HOTCs parallels that seen in vivo (Elliot et al., 1995).

Why SD is not blocked by nonspecific inhibition of Ca^{2+} channels in acute slices (Jing et al., 1993) is unknown but could be due to changes in Ca^{2+} channel function (or expression) induced by slice preparation and maintenance compared to HOTCs. For example, preparation of acute hippocampal slices constitutes a severe form of metabolic stress similar to that produced by a brief global cerebral ischemia (Paschen and Mies, 1999). Furthermore, brain slices can be hypoxic at their core (Fujii et al., 1982; Lipton and Whittingham, 1984), especially compared to HOTCs, which are only ~ 50 μ m thick (R.P. Kraig and P.E. Kunkler, unpublished observations). Since T-type Ca^{2+} channels are reversibly inhibited by hypoxia (Fearon et al., 2000) and ion channel inhibition can result in compensatory increased expression of the same (Aptowicz et al., 2002) and perhaps other ion channels, changes in channel function may influence tissue susceptibility to SD.

Pyramidal Cell Excitability and SD

Pyramidal cell excitability influences susceptibility to SD. For example, SD in vivo and in acute slices is blocked by application of NMDA receptor antagonists (Hernández-Cáceres et al., 1987; Marrannes et al., 1988; Lauritzen and Hansen, 1992; McLachlan, 1992; Nellgard and Wieloch, 1992; Psarropoulou and Avoli, 1993). SD in acute slices most often begins in the CA1 region (Janigro and Schwartzkroin, 1987). Thus, the effect of NMDA receptor antagonism on SD induction in acute slices may be due to the resultant reduced excitatory drive of CA1 cells from CA3 input. In HOTCs, however, SD most often begins in CA3 (Kunkler and Kraig, 1998), yet SD there too was blocked by NMDA receptor inhibition with APV. Since the principle excitatory drive to CA3 pyramidal neurons consists of non-NMDA receptor excitation from the DG (Casaccia-Bonofil et al., 1993; Kristensen et al., 2001), this might seem paradoxical. However, it is conceivable that NMDA receptor antagonism has a similar, key effect on the relative excitability of CA3 cells needed for SD in HOTCs. For example, some CA3 (and CA1) efferents synapse back onto CA3 pyramidal neurons in HOTCs on NMDA receptors (Debanne et al., 1995). Thus, blockade of NMDA-sensitive CA3 synapses with APV might reduce CA3 excitability sufficiently to prevent initiation of SD there. In contrast, non-

NMDA-type receptor antagonism (e.g., by CNQX) has no effect on SD initiation in vivo or in acute brain slices (Lauritzen and Hansen, 1992; Nellgard and Wieloch, 1992; Psarropoulou and Avoli, 1993) or HOTCs. Perhaps the susceptibility to SD is more tied to NMDA (instead of non-NMDA) receptor activation because of the associated greater rise in neuronal Ca^{2+} seen with the former compared to the latter (Schousboe et al., 1994; Weiss and Sensi, 2000).

SD in HOTCs is most effectively induced with brief exposure to Ringer's where NaCl is replaced by NaAc (Kunkler and Kraig, 1998). The principle effect of this anion substitution seems likely to be reversal of inhibitory postsynaptic potentials (IPSPs) (Thompson et al., 1988), which could enhance pyramidal cell excitability by increased excitatory current flow (as shown in Figure 5 by the prolonged fEPSP). Alternatively, exposure to NaAc-Ringer's could increase pyramidal cell excitability by reducing the tonic, GABA-dependent shunting of depolarizing stimuli that would otherwise be seen (Soltesz et al., 1995). Our results showing bicuculline triggering spontaneous SDs in HOTCs (before electrophysiologic function degrades to irregularly spontaneous electrographic seizures) support this latter suggestion. Bicuculline has been reported to trigger SD in acute slices (Gutierrez and Heinemann, 1999) and HOTCs (Psarropoulou and Avoli, 1993). Furthermore, while both excitatory and inhibitory synaptic transmission increase as SD begins, presynaptic inhibition transmission fails first with the onset of SD (Janigro and Schwartzkroin, 1987), suggesting that SD itself parallels the changes induced by NaAc Ringer's and bicuculline—namely a selective initial loss of inhibitory transmission with the onset of SD. Reversal (or removal) of inhibitory neurotransmission might also increase the number of pyramidal cells and associated neural volume synchronously activated by the DG electrical pulse due to reduced surround inhibition (Köhling, 2002), a change similar to that recently shown to occur in human temporal lobe epileptic tissue (Cohen et al., 2002). An increased volume of synchronously activated neural tissue has long been recognized to be essential for triggering SD (Bureš et al., 1974).

P and/or Q Ca^{2+} channels can be found in dendrites but are predominantly found along the length of the apical dendrites and somata in nerve terminals forming synapses upon CA3 neurons (Westenbroek et al., 1995). Interestingly, AgTX abolishes IPSPs generated by st. lucidum and st. oriens GABAergic cells that terminate at the perisomatic level of pyramidal cells of HOTCs (Poncer et al., 1997) and which are responsible for tonic, action-potential independent inhibition under normal circumstances in acute slices (Soltesz et al., 1995). Thus, perhaps the principle effect of P/Q channel inhibition by AgTX in our experiments is to lessen the increased excitatory input (and associated neuronal Ca^{2+} rise) provided by IPSP reversal from NaAc Ringer's exposure. Alternatively, P/Q channel inhibition could have important postsynaptic effects on excitability (and associated Ca^{2+} change) needed for triggering SD reminiscent of those known for NMDA receptor activation. Our results support the utility of further studying both possibilities. Furthermore, they indicate that further study of the balance and synchrony between pyramidal cell excitatory and inhibitory circuit function may be a key for unraveling the bases for SD.

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References

- Aitken PG, Fayuk D, Somjen GG, Turner DA. Use of intrinsic optical signals to monitor physiological changes in brain tissue slices. *Methods* 1999;18:91–103. [PubMed: 10356339]
- Aptowicz CO, Kunkler PE, Kraig RP. Endogenous control of excitability in hippocampal organ cultures. *Soc Neurosci Abs* 2002;28:95.12.
- Ault B, Evans RH, Francis AA, Oakes DJ, Watkins JC. Selective depression of excitatory amino acid induced depolarizations by magnesium ions in isolated spinal cord preparations. *J Physiol* 1980;307:413–428. [PubMed: 6259339]
- Ayata C, Shimizu-Sasamata M, Lo EH, Noebels JL, Moskowitz MA. Impaired neurotransmitter release and elevated threshold for cortical spreading depression in mice with mutations in the $\alpha 1A$ subunit of p/q type calcium channels. *Neuroscience* 2000;95:639–645. [PubMed: 10670432]
- Bahr BA. Long-term hippocampal slices: a model system for investigating synaptic mechanisms and pathological processes. *J Neurosci Res* 1995;42:294–305. [PubMed: 8583497]
- Bahr BA, Kessler M, Rivera S, Vanderklish PW, Hall RA, Mutneja MS, Gall C, Hoffman KB. Stable maintenance of glutamate receptors and other synaptic components in long-term hippocampal slices. *Hippocampus* 1995;5:425–439. [PubMed: 8773255]
- Basarsky TA, Duffy SN, Andrew RD, MacVicar BA. Imaging spreading depression and associated intracellular calcium waves in brain slices. *J Neurosci* 1998;18:7189–7199. [PubMed: 9736642]
- Beck H, Steffens R, Heinemann U, Elger CE. Properties of voltage-activated Ca^{2+} currents in acutely isolated human hippocampal granule cells. *J Neurophysiol* 1997;77:1526–1537. [PubMed: 9084617]
- Berridge MJ. Neuronal calcium signaling. *Neuron* 1998;21:13–26. [PubMed: 9697848]
- Bureš, J.; Burešová, O.; Křivánek, J. The mechanism and applications of Leão's spreading depression of electroencephalographic activity. New York: Academic Press; 1974.
- Casaccia-Bonnel P, Benedikz E, Rai R, Bergold PJ. Excitatory and inhibitory pathways modulate kainate excitotoxicity in hippocampal slice cultures. *Neurosci Lett* 1993;154:5–8. [PubMed: 8361647]
- Cohen I, Navarro V, Clemenceau S, Baulac M, Miles R. On the origin of interictal activity in human temporal lobe epilepsy in vitro. *Science* 2002;298:1418–1421. [PubMed: 12434059]
- Debanne D, Guerinéau NC, Gähwiler BH, Thompson SM. Physiology and pharmacology of unitary synaptic connections between pairs of cells in areas CA3 and CA1 of rat hippocampal slice cultures. *J Neurophysiol* 1995;73:1282–1294. [PubMed: 7608771]
- Elliott EM, Malouf AT, Catterall WA. Role of calcium channel transients in hippocampal CA3 neurons. *J Neurosci* 1995;15:6433–6444. [PubMed: 7472406]
- Fearon IM, Randall AD, Perez-Reyes E, Peers C. Modulation of recombinant T-type Ca^{2+} channels by hypoxia and glutathione. *Pflugers Arch* 2000;441:181–188. [PubMed: 11211102]
- Footitt DR, Newberry NR. Cortical spreading depression induces an LTP-like effect in the rat neocortex in vitro. *Brain Res* 1998;781:339–342. [PubMed: 9507183]
- Fujii T, Baumgartl H, Lubbers DW. Limiting section thickness of guinea pig olfactory cortical slices studied from tissue pO_2 values and electrical activities. *Pflugers Arch* 1982;393:83–87. [PubMed: 7088686]
- Gähwiler BH, Capogna M, Debanne D, McKinney RA, Thompson SM. Organotypic slice cultures: a technique has come of age. *Trends Neurosci* 1997;20:471–477. [PubMed: 9347615]
- Gingrich JR, Roder J. Inducible gene expression in the nervous system of transgenic mice. *Annu Rev Neurosci* 1998;21:377–405. [PubMed: 9530501]
- Gorji A, Scheller D, Tegtmeir F, Köhling R, Straub H, Speckmann EJ. $NiCl_2$ and amiloride induce spreading depression in guinea pig hippocampal slices. *Cephalalgia* 2000;20:740–747. [PubMed: 11167905]
- Gutierrez R, Heinemann U. Synaptic reorganization in explanted cultures of rat hippocampus. *Brain Res* 1999;815:304–316. [PubMed: 9878801]
- Hansen AJ, Olsen CE. Brain extracellular space during spreading depression and ischemia. *Acta Physiol Scand* 1980;108:355–365. [PubMed: 7415848]

- Harris KM, Jensen FE, Tsao B. Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. *J Neurosci* 1992;12:2685–2705. [PubMed: 1613552]
- Hashimoto M, Yoshimasa T, Sato T, Kawahara H, Naganon O, Hirakawa M. Dynamic changes in NADH fluorescence images and NADH content during spreading depression in the cerebral cortex of gerbils. *Brain Res* 2000;872:294–300. [PubMed: 10924711]
- Haugland, RP. Handbook of fluorescent probes and research products. Eugene, OR: Molecular Probes; 2002.
- Hernández-Cáceres J, Macías-González R, Brožek G, Bures J. Systemic ketamine blocks cortical spreading depression but does not delay the onset of terminal anoxic depolarization in rats. *Brain Res* 1987;437:360–364. [PubMed: 3435842]
- Herreras O, Somjen GG. Analysis of potential shifts associated with recurrent spreading depression and prolonged unstable SD induced by microdialysis of elevated K^+ in hippocampus of anesthetized rats. *Brain Res* 1993;610:283–294. [PubMed: 8319090]
- Hille B. Charges and potentials at the nerve surface. Divalent ions and pH. *J Gen Physiol* 1968;51:221–236. [PubMed: 5641636]
- Janigro D, Schwartzkroin PA. Dissociation of the IPSP and response to GABA during spreading depression-like depolarizations in hippocampal slices. *Brain Res* 1987;404:189–200. [PubMed: 3032331]
- Jing J, Aitken PG, Somjen GG. Role of calcium channels in spreading depression in rat hippocampal slices. *Brain Res* 1993;604:251–259. [PubMed: 8384511]
- Knöpfel T, Gähwiler BH. Activity-induced elevations of intracellular calcium concentration in pyramidal and nonpyramidal cells of CA3 region of rat hippocampal slice cultures. *J Neurophysiol* 1992;68:961–963. [PubMed: 1432060]
- Köhling R. GABA becomes exciting. *Science* 2002;298:1350–1352. [PubMed: 12434044]
- Kovacs R, Schuchmann S, Gabriel S, Kardos J, Heinemann U. Ca^{2+} signaling and changes of mitochondrial function during low- Mg^{2+} -induced epileptiform activity in organotypic hippocampal slice cultures. *Eur J Neurosci* 2001;13:1311–1319. [PubMed: 11298791]
- Kraig, RP. Astrocytic acid-base homeostasis in cerebral ischemia. In: Schurr, A.; Rigor, BM., editors. *Cerebral ischemia and resuscitation*. Boca Raton, FL: CRC Press; 1990. p. 89-99.
- Kraig, RP.; Kunkler, PE. Spreading depression—a teleologic means for self-protection from brain ischemia. In: Chan, PH., editor. *Cerebrovascular disease. Twenty-second Princeton Research conference*; New York: Cambridge University Press; 2002. p. 142-157.
- Kraig RP, Nicholson C. Extracellular ionic variations during spreading depression. *Neuroscience* 1978;3:1045–1059. [PubMed: 745780]
- Kraig, RP.; Lascola, CD.; Caggiano, AO. Glial response to brain ischemia. In: Ransom, B.; Kettenmann, H., editors. *Neuroglial cells*. New York: Oxford University Press; 1995. p. 964-976.
- Kristensen BW, Noraberg J, Zimmer J. Comparison of excitotoxic profiles of ATPA, AMPA, KA and NMDA in organotypic hippocampal slice cultures. *Brain Res* 2001;917:21–44. [PubMed: 11602227]
- Kunkler PE, Kraig RP. Calcium waves precede electrophysiological changes of spreading depression in hippocampal organ cultures. *J Neurosci* 1998;18:3416–3425. [PubMed: 9547248]
- Kunkler PE, Kraig RP. Reactive astrogliosis from excitotoxic injury in hippocampal organ culture parallels that seen in vivo. *J Cereb Blood Flow Metab* 1997;17:26–43. [PubMed: 8978384]
- Lauritzen M, Hansen AJ. The effect of glutamate receptor blockade on anoxic depolarization and cortical spreading depression. *J Cereb Blood Flow Metab* 1992;12:223–229. [PubMed: 1312539]
- Leão AAP. Spreading depression of activity in the cerebral cortex. *J Neurophysiol* 1944;7:359–390.
- Lipton, P.; Whittingham, TS. Energy metabolism and brain slice function. In: Dingledine, R., editor. *Brain slices*. New York: Plenum; 1984. p. 113-153.
- Llinás R. The intrinsic electrophysiological properties of mammalian neurons: insights into the central nervous system function. *Science* 1988;242:1654–1664. [PubMed: 3059497]
- Llinás R, Hess R. Tetrodotoxin-resistant dendritic spikes in avian Purkinje cells. *Proc Natl Acad Sci USA* 1976;73:2520–2523. [PubMed: 1065905]

- Marrannes R, Willems R, De Prins E, Wauquier A. Evidence for a role of the N-methyl-D-aspartate (NMDA) receptor in cortical spreading depression in the rat. *Brain Res* 1988;457:226–240. [PubMed: 2851364]
- Marrannes, R.; De Prins, E.; Fransen, J.; Clincke, G. Neuropharmacology of spreading depression. In: Lehmenkühler, A.; Grotemeyer, K-H.; Tegtmeier, F., editors. *Migraine: basic mechanisms and treatment*. Baltimore, MD: Urban & Schwarzenberg; 1993. p. 431-443.
- McLachlan RS. Suppression of spreading depression of Leão in neocortex by an N-methyl-D-aspartate receptor antagonist. *Can J Neurol Sci* 1992;19:487–491. [PubMed: 1358423]
- McKinney RA, Capogna M, Dürr R, Gähwiler BH, Thompson SM. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat Neurosci* 1999;2:44–49. [PubMed: 10195179]
- Miller RJ. Multiple calcium channels and neuronal function. *Science* 1987;235:46–52. [PubMed: 2432656]
- Mogul DJ, Fox AP. Evidence for multiple types of Ca^{2+} channels in acutely isolated hippocampal CA3 neurons of the guinea pig. *J Physiol* 1991;433:259–281. [PubMed: 1668752]
- Mori S, Miller WH, Tomita T. Muller cell function during spreading depression in frog retina. *Proc Natl Acad Sci USA* 1976;73:1351–1354. [PubMed: 1083528]
- Müller M, Somjen GG. Inhibition of major cationic inward currents prevents spreading depression-like hypoxic depolarization in rat hippocampal tissue slices. *Brain Res* 1998;812:1–13. [PubMed: 9813218]
- Nellgard B, Wieloch T. NMDA-receptor blockers but not NBQX, an AMPA-receptor antagonist, inhibit spreading depression in the rat brain. *Acta Physiol Scand* 1992;146:497–503. [PubMed: 1283483]
- Nicholson C, ten Bruggencate G, Steinberg R, Stöckle H. Calcium modulation in brain extracellular microenvironment demonstrated with ion-selective micropipette. *Proc Natl Acad Sci USA* 1977;74:1287–1290. [PubMed: 265573]
- Nicholson C, ten Bruggencate G, Stöckle H, Steinberg R. Calcium and potassium changes in extracellular microenvironment of cat cerebellar cortex. *J Neurophysiol* 1978;41:1026–1039. [PubMed: 681986]
- Nicholson C, Phillips JM. Ion diffusion modified by tortuosity and volume fraction in the extracellular microenvironment of the rat cerebellum. *J Physiol* 1981;321:225–257. [PubMed: 7338810]
- Nicholson, C.; Phillips, JM.; Tobias, C.; Kraig, RP. Extracellular potassium, calcium and volume profiles during spreading depression. In: Syková, E.; Hník, P.; Vyklíček, L., editors. *Ion-selective microelectrodes and their use in excitable tissues*. New York: Plenum; 1981. p. 211-223.
- Paschen W, Mies G. Effect of induced tolerance on biochemical disturbances in hippocampal slices from gerbil during and after oxygen/glucose deprivation. *NeuroReport* 1999;10:1417–1421. [PubMed: 10380956]
- Pauwels PJ, Leyse JE, Laduron PM. [^3H]-batrachotoxinin A 20- α -benzoate binding to sodium channels in rat brain: characterization and pharmacological significance. *Eur J Pharmacol* 1986;124:291–298. [PubMed: 2426120]
- Petr MJ, Wurster RD. Determination of in situ dissociation constant for Fura-2 and quantification of background fluorescence in astrocyte cell line U373-MG. *Cell Calcium* 1997;21:233–240. [PubMed: 9105732]
- Poncer JC, McKinney RA, Gähwiler BH, Thompson SM. Either N- or P-type calcium channels mediate GABA release at distinct hippocampal inhibitory synapses. *Neuron* 1997;18:463–472. [PubMed: 9115739]
- Pozzo-Miller LD, Petroszino JJ, Mahanty NK, Connor JA. Optical imaging of cytosolic calcium, electrophysiology, and ultrastructure in pyramidal neurons of organotypic slice cultures from rat hippocampus. *Neuroimage* 1993;1:109–120. [PubMed: 9343562]
- Pozzo-Miller LD, Connor JA, Andrews SB. Microheterogeneity of calcium signaling in dendrites. *J Physiol* 2000;525:53–61. [PubMed: 10811724]
- Psarropoulou C, Avoli M. 4-Aminopyridine-induced spreading depression episodes in immature hippocampus: development and pharmacological characteristics. *Neuroscience* 1993;55:57–68. [PubMed: 8102481]
- Puppels, GJ.; Coremans, JMCC.; Bruining, HA. In vivo semiquantitative NADH-fluorescence imaging. In: Mason, WT., editor. *Fluorescent and luminescent probes for biological activity*. San Diego, CA: Academic Press; 1999. p. 456-466.

- Richter F, Ebersberger A, Schaible HG. Blockade of voltage-gated channels in rat inhibits repetitive cortical spreading depression. *Neurosci Lett* 2002;334:123–126. [PubMed: 12435487]
- Rose CR, Konnerth A. Stores not just storage: intracellular calcium release and synaptic plasticity. *Neuron* 2001;31:519–522. [PubMed: 11545711]
- Schmitt M, Kunkler PE, Aptowicz CO, Kraig RP. Seizures and neuronal loss need not be expected responses of maturing hippocampal organ cultures. *Soc Neurosci Abs* 2002;28:95.6.
- Schousboe A, Frandsen A, Wahl P, Krogsaard-Larsen P. Neurotoxicity and excitatory amino acid antagonists. *Neurotoxicology* 1994;15:477–481. [PubMed: 7854581]
- Schwartzkroin PA, Slawsky M. Probable calcium spikes in hippocampal neurons. *Brain Res* 1977;135:157–161. [PubMed: 912429]
- Siesjo BK, Bengtsson F. Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: a unifying hypothesis. *J Cereb Blood Flow Metab* 1989;9:127–140. [PubMed: 2537841]
- Smetters D, Majewska A, Yuste R. Detecting action potentials in neuronal populations with calcium imaging. *Methods* 1999;18:215–221. [PubMed: 10356353]
- Snow RW, Taylor CP, Dudek FE. Electrophysiological and optical changes in slices of rat hippocampus during spreading depression. *J Neurophysiol* 1983;50:561–572. [PubMed: 6311993]
- Soltesz I, Smetters DK, Mody I. Tonic inhibition originates from synapses close to the soma. *Neuron* 1995;14:1273–1283. [PubMed: 7605636]
- Somjen GG. Electrogenesis of sustained potentials. *Prog Neurobiol* 1973;1:201–257. [PubMed: 4591394]
- Somjen GG. Mechanisms of spreading depression and hypoxic spreading depression-like depolarizations. *Physiol Rev* 2001;81:1065–1096. [PubMed: 11427692]
- Sonn J, Mayevsky A. Effects of brain oxygenation on metabolic, hemodynamic, ionic and electrical responses to spreading depression in the rat. *Brain Res* 2000;882:212–216. [PubMed: 11056202]
- Stoppini L, Buchs PA, Muller D. A simple method for organotypic cultures of the nervous system. *J Neurosci Methods* 1991;37:173–182. [PubMed: 1715499]
- Streit P, Thompson SM, Gähwiler BH. Anatomical and physiological properties of GABAergic neurotransmission in organotypic slice cultures of rat hippocampus. *Eur J Neurosci* 1989;1:603–615. [PubMed: 12106118]
- Takagi H, Takashima M, Liou SY. Effect of KB-2796, a novel calcium channel, on spreading depression in rat hippocampal slices. *Nippon Yakurigaku Zasshi* 1998;111:309–316. [PubMed: 9666486]
- Tang CM, Presser F, Morad M. Amiloride selectively blocks the low threshold (T) calcium channel. *Science* 1988;240:213–215. [PubMed: 2451291]
- Thompson SM, Deisz RA, Prince DA. Relative contributions of passive equilibrium and active transport to the distribution of chloride in mammalian cortical neurons. *J Neurophysiol* 1988;60:105–124. [PubMed: 3404212]
- Usowicz M, Sugimori M, Cherksey B, Llinás R. P-type calcium channels in the somata and dendrites of adult cerebellar Purkinje cells. *Neuron* 1992;9:1185–1199. [PubMed: 1281419]
- Wauquier A, Ashton D, Marrannes R. The effects of flunarizine in experimental models related to the pathogenesis of migraine. *Cephalalgia* 1985;5(suppl 2):119–123. [PubMed: 4016924]
- Weiss JH, Sensi SL. $\text{Ca}^{2+}\text{Zn}^{2+}$ permeable AMPA or kainate receptors: possible key factors in selective neurodegeneration. *TINS* 2000;23:365–371. [PubMed: 10906800]
- Westenbroek RE, Sakurai T, Elliot EM, Hell JW, Starr TVB, Snutch TP, Catterall WA. Immunohistochemical identification and cellular distribution of the $\alpha 1A$ subunits of brain calcium channels. *J Neurosci* 1995;15:6403–6418. [PubMed: 7472404]
- Wheeler DB, Randall A, Tsien RW. Roles of N-type and Q-type Ca^{2+} channels in supporting hippocampal synaptic transmission. *Science* 1994;264:107–111. [PubMed: 7832825]
- Wong RKS, Prince DA, Basbaum AI. Intradendritic recordings from hippocampal neurons. *Proc Natl Acad Sci USA* 1979;76:986–990. [PubMed: 284423]
- Zimmer J, Gähwiler BH. Cellular and connective organization of slice cultures of the rat hippocampus and fascia dentate. *J Comp Neurol* 1984;228:432–446. [PubMed: 6148364]

FIGURE 1.

Evoked field potential changes associated with spreading depression in hippocampal organotypic cultures. DC potential records evoked under normal conditions (A) and those triggered with induction of spreading depression (SD) (B). Upper record for each set shows slow potential changes and lower records show associated fast, field potential changes. In each case, field potentials were initially evoked every second (records 1–10) and then every 10 s (records 10–20) using a bipolar electrical stimulus delivered to the dentate gyrus (DG). Interstitial recordings were made in the CA3 pyramidal cell body layer. Little slow potential change occurred under normal conditions and no change occurred in evoked field potentials with this stimulation paradigm (i.e., records 1–20 in A). In contrast, after 2-min exposure to

NaAc-based Ringer's solution, a single bipolar stimulus triggered SD as evidenced by the large interstitial change in the slow, DC potential (upper record of B) and associated loss of evoked field potentials (records 2–11 in B) and then a progressive return of the pyramidal cell field excitatory postsynaptic potential (fEPSP) and then population spike after SD (records 12–20 in B). The SD triggering pulse occurs with increased excitability of the CA3 pyramidal cells (record 1 in B) that is evidenced by the initiation of 3 population spikes, in this example, and a markedly prolonged EPSP. For emphasis of this evoked field potential change, the first records of each condition set are darkened. Voltage calibration bar applies to all records. Time calibration is 10 s for slow, upper records and 10 ms for lower, fast records.

FIGURE 2.

Spreading depression propagation in hippocampal organotypic cultures (HOTCs). Cellular voltage changes (9 images above) and interstitial DC potential changes (two records below) were monitored simultaneously to illustrate the third classical electrophysiologic characteristic of spreading depression (SD), slow propagation of depolarization, in HOTCs. Cellular depolarization was monitored in HOTCs stained with the voltage-sensitive dye RH795 and is evident as a darkened zone that began (image 1) at dentate gyrus (DG)-CA3. This area progressed in size (images 2–4) before progressively returning toward normal (images 5–9). As cellular polarization returned toward normal, two distinct bands of depolarization persisted as other areas repolarized (images 5–9). Schematic in image 1 shows relative positions of

bipolar stimulating electrode in the DG and recording micropipettes in CA3 and CA1. DC records from these recording microelectrodes are shown in bottom records. All 9 images were taken during the depolarizing phase of SD as noted by the dots on the CA3 record. SD was initiated by a single electrical pulse (-10 -mV drop in CA3 interstitial trace) following a 90-s perfusion with NaAc Ringer's. Images are shown at 5-s intervals. Images are 2.33 mm across. Thus, RH795 fluorescent depolarization changes show a SD propagation velocity of 2.18 mm/min.

FIGURE 3.

Neuronal Ca^{2+} transients during spreading depression (SD) in hippocampal organotypic cultures (HOTCs). A typical CA3 pyramidal cell filled with fura-2 using a sharp microelectrode is shown (A) with the three areas quantified (more distal basilar dendrites (D2), proximal basilar dendrites (D1), and soma (S). Small white arrows note two, adjacent pyramidal cell bodies that were filled with fura-2 from a single cellular penetration and injection. This most likely reflects the high degree of gap junctional connections found between these cells in HOTCs (Kraig and Kunkler, 2000), a finding consistent with that seen in vivo. Pyramidal cell Ca^{2+} changes from SD showed three distinct peaks (marked 1, 2, 3) that were most evident in the dendrites (B). The relative contribution of each of the three peak changes in pyramidal cell Ca^{2+} from SD

are shown as percentage peak changes (C). The first peak Ca^{2+} change occurred with the triggering pulse of SD and was of intermediate magnitude. The second occurred with the start of SD and was significantly (*) greater than either of the other two peaks in the dendrites ($P < 0.001$), but only significantly greater than the first peak ($P < 0.05$) in the soma. This is evident by the lower and slower rise and fall of the fura-2 ratio within the soma (green curve in B, histograms in C). Ratiometric (340/380) Ca^{2+} levels were sampled at 1.5 Hz. DC potential change from SD recorded in adjacent interstitial space is shown in D.

FIGURE 4.

Effect of ω -agatoxin-IVA on CA3 pyramidal cell Ca^{2+} changes. Records show CA3 pyramidal cell Ca^{2+} changes (black curves) and nearby (i.e., 100–200 μm away) interstitial DC potential change (white curves) before (A) and after (B) exposure to AgTX. Calibrations are equivalent for each record set. Records in A show no neuronal Ca^{2+} change was evident after single bipolar stimulus to the dentate gyrus (DG) that evoked a field potential in the CA3 area (small sharp positive and minimal slow negative DC change). Exposure to 175 nM ω -agatoxin-IVA for 30 min prevented spreading depression (SD) from combined exposure to NaAc Ringer's and a single bipolar electrical stimulus to the DG. This stimulation did, however, trigger a small change in Ca^{2+} consistent with the first small peak previously seen that preceded spreading depression (SD) (Fig. 3).

FIGURE 5.

Effect of ω -agatoxin-IVA on NaAc Ringer's induced CA3 hyperexcitability in hippocampal organotypic cultures. Records show typical evoked field potentials in CA3 from single dentate gyrus (DG) bipolar stimulus. Upper record shows field potential under normal conditions with single population spike followed by large field excitatory postsynaptic potential (fEPSP) occurring afterward. Middle record shows that 2-min exposure to NaAc Ringer's solution caused an increase in pyramidal cell excitability from this same DG bipolar stimulus. This was evident by a significant increase ($P < 0.001$) in the time to peak fEPSP compared to normals. Exposure to ω -agatoxin-IVA (lower record) reduced this NaAc-induced increased excitability of pyramidal cells to a nonsignificant difference from control conditions.

TABLE 1**Ca²⁺ Channel Antagonist Blockade on SD in HOTCs**

Sample	Concentration	% Blockade of SD ^a
Control		0 (10)
Ni ²⁺	(500 μ M)	22 (9)
Ni ²⁺	(1 mM)	100 (5)
Cd ²⁺	(50 μ M)	100 (9)
APV	(50 μ M)	83 (6)
CNQX	(20 μ M)	0 (5)
CNQX	(100 μ M)	100 (5)
Nifedepine	(10 μ M)	0 (5)
ω -Conotoxin GVIA	(2 μ M)	0 (6)
ω -Agatoxin-IVA	(20 nM)	0 (6)
ω -Agatoxin-IVA	(175 nM)	92 (13)

SD, spreading depression; HOTCs, hippocampal organotypic cultures; APV, amino-phosphonovaleric acid; CNQX, cyanonitroquinoxaline.

^aNumber of cultures in parentheses.