Novel Form of Spreading Acidification and Depression in the Cerebellar Cortex Demonstrated by Neutral Red Optical Imaging

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Chen, G., C. L. Hanson, R. L. Dunbar, and T. J. Ebner. Novel form of spreading acidification and depression in the cerebellar cortex demonstrated by neutral red optical imaging. J. Neurophysiol. 81: 1992–1998, 1999. A novel form of spreading acidification and depression in the rat cerebellar cortex was imaged in vivo using the pH-sensitive dye, Neutral red. Surface stimulation evoked an initial beam of increased fluorescence (i.e., decreased pH) that spread rostrally and caudally across the folium and into neighboring folia. A transient but marked suppression in the excitability of the parallel fiber-Purkinje cell circuitry accompanied the spread. Characteristics differentiating this phenomenon from the spreading depression of Leao include: high speed of propagation on the surface (average of 450 µm/s), stable extracellular DC potential, no change in blood vessel diameter, and repeatability at short intervals. This propagating acidification constitutes a previously unknown class of neuronal processing in the cerebellar cortex.

INTRODUCTION

Propagating waves of excitatory and inhibitory activity across neuronal populations are of particular interest due to their roles in neuronal signaling as well as pathophysiology. Calcium waves have been shown to occur in various neuronal-glial in vitro preparations and have been hypothesized to act as a signaling mechanism (Cornell-Bell et al. 1990; Newman and Zahs 1997). Spreading depression of Leao is a slowly propagating wave that results in a dramatic extinction of all neuronal activity (for review, see Lauritzen and Nicholson 1988; Leao 1944; Ochs 1962). This “classical” form of spreading depression is characterized by a large negative shift in the extracellular DC potential, relatively long refractory period, and large ionic shifts including a transient increase in extracellular K+ concentration. Classical spreading depression may play a role in several pathophysiological processes including migraine headaches and ischemia (Lauritzen and Nicholson 1988; Somjen et al. 1992).

Extra- and intracellular pH are coupled closely to neuronal activity (Chen et al. 1998; Chesler 1990; Chesler and Craig, 1989; Grichtchenko and Chesler 1994a,b; Kraig et al. 1983). Stimulation of the cerebral or cerebellar cortex evokes an initial alkaline shift followed by an acidic shift in the extracellular space. Intracellularly, this sequence is reversed; neurons undergo an acidic shift while glial cells become alkaline. The pH-sensitive dye, Neutral red, can be used to monitor the spatial and temporal characteristics of pH shifts in vivo and map neuronal activation (Chen et al. 1996, 1998). This report demonstrates that electrical stimulation of the surface of the cerebellar cortex stained with Neutral red evokes an optical signal that propagates at high speed and is accompanied by a transient but powerful depression of parallel fibers and their postsynaptic targets. This novel form of spreading acidification and depression differs fundamentally from the spreading depression of Leao and other previously described wave-like phenomenon.

METHODS

Details of the preparation are provided in previous publications (Chen et al. 1996, 1998). Briefly, adult Sprague-Dawley rats of either sex (200–400 g) were anesthetized by an intramuscular injection of a solution of ketamine (60 mg/kg), xylazine (3 mg/kg), and acepromazine (1.2 mg/kg). The animals were respirated artificially. After exposing Crus I and II of the cerebellar surface, Neutral red (8–9 mM in rat Ringer solution) was applied topically for 2–3 h. Surface folia were imaged using a CCD camera with modified Zeiss epi-fluorescence optics. After washout of the dye, sequential epi-fluorescence images (excitation at 550 ± 10 nm, emission >620 nm) of the stained cortex were acquired before (control), during, and after (stimulation image) a train of surface electrical stimulation with a tungsten micro-electrode. Acquisition time for an individual frame was 100 ms with a slight delay between frames. Subtraction of a single control image from a stimulation image was used as shown in Fig. 1. No averaging of the images was done. Conventional electrophysiological techniques and glass micropipettes were used to record extracellular field potentials evoked by surface stimulation and to monitor extracellular DC potentials.

In some experiments, the voltage-sensitive dye, RH-795, was used to stain the cerebellar cortex and the optical response to surface stimulation imaged. RH-795 was chosen because it yields a large optical response to surface stimulation in vivo and has excitation-emission characteristics similar to Neutral red (Ebner and Chen 1995). The exposed cortex was bathed in RH-795 (0.3 mg/ml) for 2 h. After washout of the dye, epi-fluorescence images (excitation at 546 ± 10 nm, emission >590 nm) were acquired. To detect the much weaker optical signals obtained from the cerebellum stained with RH-795 (Ebner and Chen 1995), frame averaging (30–100) was required. Specifically, 30–100 pairs of images without and with surface stimulation were obtained (exposure time of 100 ms) and subtracted. The resultant set of “difference images” was averaged.

Classical spreading depression also was imaged in the Neutral-red-stained cerebellar cortex. Because spreading depression in the rat...
The cerebellar cortex is difficult to evoke (Nicholson and Kraig 1975; Nicholson et al. 1978) and due to the inhibitory effects of ketamine on the generation of spreading depression (Gorelova et al. 1987; Lauritzen et al. 1988), several changes to the experimental protocol were required. The animals were anesthetized with pentobarbital (50 mg/kg ip), and the cerebellar cortex was conditioned by bathing in a modified Ringer solution that contained 123 mM sodium benzoate, completely replacing the NaCl (Nicholson and Kraig 1975; Tobiasz and Nicholson 1982). To evoke classical spreading depression, KCl was injected using a picospritzer pressure injection system (Medical Systems, Greenvale, NY). A glass microelectrode with a 1- to 3-μm-diameter tip was filled with 0.5 M KCl, and ~5 nl was injected over 1–3 s. A negative holding pressure was applied to the electrode when not injecting. Optical images and the extracellular DC potential were recorded in relation to the KCl injection.

RESULTS

Electrical stimulation of the surface with a train of 100 pulses initially evoked a narrow beam of increased fluorescence along the long axis of the folium (Fig. 1A). An increase in fluorescence reflects a decrease in pH, and this beam-like signal is due to the activation of parallel fibers and their postsynaptic targets (Chen et al. 1998). During the first 2.7 s of the stimulus train, the fluorescence increase in the center of Crus Ia was 0.5% (ΔF/F) and remained confined to a restricted beam. The increase in fluorescence began to rapidly spread rostral-caudally across the surface of the folium; 2.85 s from the onset of stimulation. At 3.45 s, the optical signal reached the anterior edge (position b in Fig. 1B). During the spread, the intensity of the signal at the center of Crus Ia rapidly increased to 25% (ΔF/F) and remained elevated for the next 50 s (Fig. 1C). After a delay of 12.7 s, the increase in fluorescence spread to the anterior edge (position c in Fig. 1B) of the neighboring folia, Crus Ib (16.05 s in Fig. 1A). The optical signal then spread rapidly across the surface of Crus Ib and reached its posterior boundary within 16.95 s (position d).
increase in fluorescence in Crus Ib persisted throughout the remainder of the observation time (Fig. 1C). The optical signal continued to spread and arrived at Crus IIa, which was just visible in the lower right corner of the field of view, at 33.15 s (position e). The long delays in the arrival of the optical signal in Crus Ib and II and the rapid but persistent increases in fluorescence after reaching these folia are demonstrated in Fig. 1C.

The speed at which the acidification spread across the folium is in sharp contrast to other forms of propagated activity in the CNS. Calcium waves propagate at $\sim 25 \mu m/s$ (Newman and Zahs 1997), whereas classical spreading depression in the cerebral cortex propagates at 25–125 $\mu m/s$ with the highest speed of 150 $\mu m/s$ reported in the rat cerebellar cortex (Leao 1944; Nicholson et al. 1978; Somjen et al. 1992). For the example shown in Fig. 1, the average speed was 470 $\mu m/s$ with a peak speed of 1,100 $\mu m/s$ on the surface of the folia (a to b’ and a to b on Crus Ia and from c to d on Crus Ib). In 14 animals in which the speed of the optical wavefront was calculated, the average speed on the cerebellar surface was 450 $\pm 80$ (SD) $\mu m/s$ with an average peak speed of 940 $\pm 220$ $\mu m/s$. The speed of propagation within the sulcus was 180 $\mu m/s$ based on the assumption that the speed followed the most direct path as estimated in Fig. 1D (from b to c and d to e). However, the actual path taken by the spread is unknown and the speed in the sulcus may be underestimated. In the experiment shown in Fig. 1, the optical signal did not spread into lobulus simplex (SL), the most anterior folium in the field of view. As shown in Fig. 1D, the sulcus between Crus Ia and SL was relatively deep, suggesting that the distance over which the spread occurs may be limited to a few centimeters. Spread of the optical signal was observed in 40 of 73 animals. Stimulation parameters were an important determinant of whether spread was evoked. Increasing stimulation frequency (2–20 Hz), duration (2–30 s), and intensity (75–300 $\mu A$) increased the likelihood of evoking the spread.

The gradients in the optical response both along the stimulation evoked parallel fiber beam and perpendicular to the beam are shown in Fig. 1, E–G, at four different times in the evolution of the response. At no time in the course of the spread was there an obvious gradient in the optical response along the beam (F). In contrast, a very large gradient in the optical response occurred perpendicular to the beam (Fig. 1G). The amplitude of the optical response was largest on beam, decreasing as a function of the distance from the beam. Furthermore, the optical response was symmetric relative to the beam throughout the course of the spread. The second peak of activity at 2,000 $\mu A$ was observed in 40 of 73 animals. Stimulation parameters were an important determinant of whether spread was evoked. Increasing stimulation frequency (2–20 Hz), duration (2–30 s), and intensity (75–300 $\mu A$) increased the likelihood of evoking the spread.

The excitability of the cerebellar circuitry in relationship to the spread of acidification was evaluated by recording both the optical signals and the extracellular field potentials (Fig. 2) evoked by a second surface stimulating electrode. Two stimulation electrodes were placed on the surface (Fig. 2A). The first electrode (S1) was used to evoke the spread of optical activity using a train of stimulation (150–$\mu A$, 15–$\mu s$ pulses at 10 Hz for 15 s). The second, laterally placed electrode (S2) was used to stimulate the parallel fibers at 2-s intervals with a single pulse (150 $\mu A$, 150 $\mu s$) and at the same time an optical image was captured. The resultant parallel fiber volley (positive-negative-positive deflection, $P_1/N_1/P_2$ components) and
postsynaptic response (longer latency negative deflection, N_2 component) were recorded “on beam” relative to the second stimulation electrode (Fig. 2A). The amplitude of P_1 to N_1 was used as a measure of parallel fiber excitability and the amplitude of N_2 as a measure of the postsynaptic response. The stimulus train initially evoked a narrow beam of increased fluorescence (b in Fig. 2A) that spread across the entire folium (c-d). The increase in fluorescence peaked at 25.8 s after the onset of stimulation and decreased during the next 50 s to a level \( \%\) above baseline (Fig. 2A and B).

Examination of the extracellular field potentials (Fig. 2C) revealed a transient but complete suppression of the parallel fiber (P_1/N_1) and postsynaptic component (N_2) before and during the peak of the optical response. Suppression of the parallel fiber volley developed rapidly and lasted \( \pm\) 20 s (Fig. 2C and D). The postsynaptic component (N_2) also was suppressed completely and took longer to recover. The postsynaptic component was abolished for \( \pm\) 120 s after the onset of stimulation, recovering to 95% of baseline at 420 s. In six of seven experiments in which the field potentials were monitored, there was complete suppression of P_1/N_1 and N_2 components during the peak of the spread of the optical signal.

In seven experiments, the cerebellar cortex was stained with the voltage-sensitive dye, RH-795, and the epi-fluorescence optical response evoked by surface stimulation was imaged. Because the optical signals obtained with voltage sensitive dyes are very small, frame averaging was required. The optical response consisted of a “beam” of decreased fluorescence (Fig. 3C) consistent with our previously published findings (Elias et al. 1993) and with the fact that depolarization leads to a decrease in epifluorescence in this class of styryl dyes (Ebner and Chen 1995). In no instance was a spread of the optical response observed using RH-795.

Is the spreading acidification and depression described in this report the spreading depression of Leao? Both the high speed of propagation on the cerebellar surface and the relatively brief suppression of the parallel fiber volley suggest that the spread of acidification and associated decrease in excitability is a unique phenomenon that has not been described previously. Four additional experimental observations confirm this conclusion. First, a hallmark of classical spreading depression is an extracellular DC shift of \(-15\) to \(-35\) mV that lasts up to several minutes (Kraig and Nicholson 1978; Lauritzen and Nicholson 1988; Leao 1944). In 12 experiments in which the extracellular DC potential was monitored, no shift in the extracellular potential occurred (Fig. 4A). Second, decreased cerebral blood flow follows a period of increased blood flow and vessel dilation in the spreading depression of Leao (Lauritzen and Nicholson 1988; Somjen et al. 1992). Blood vessel dilatation did not occur at the stimulation parameters used in this study. In seven experiments, 26 blood vessels with diameters ranging from 50–180 \( \mu\)m were measured before and during the spread of the optical signal (Fig. 4B). No significant change in blood vessel diameter was observed (\( P = 0.71, \) Student’s paired \( t\)-test). It should be noted that increases in blood vessel diameter on the cerebellar surface do occur with stimulus trains of much higher frequency (30 Hz) and duration (40–60 s) (Iadecola et al. 1997). Third, classical spreading
depression is characterized by an absolute refractory period of 120–180 s during which it is not possible to evoke a second event (Ochs 1962). In this study, a second and third spread of the optical signal could be evoked within 90 s (shortest interval tested) of each other (Fig. 4C). Furthermore, parallel fiber excitability returned to normal within 20 s of stimulation onset (Fig. 2D), a much shorter recovery time than observed with classical spreading depression (Ochs 1962).

To directly compare the spreading acidification described in this paper with classical spreading depression, KCl was pressure injected into the folium (Fig. 5). As described in METHODS, ketamine had to be replaced with pentobarbital and the Cl⁻ in the Ringer solution replaced with benzoate ion (Nicholson and Kraig 1975; Tobiasz and Nicholson 1982). As shown in Fig. 5A, microinjection of KCl evoked a strong optical signal that spread outward from the injection site at a speed of 109 μm/s, ~5–10 times slower than the spreading acidification of Fig. 1. The spread of the optical signal was accompanied by an extracellular DC shift of −15 mV (Fig. 5B). Similar observations were made in five animals.

**DISCUSSION**

What is the spreading phenomenon described in the study? The lack of a DC shift, high speed, no change in blood vessel diameter, and a short recovery period demonstrate that the spread of acidification evoked is not the spreading depression of Leao. Additional observations support this conclusion. First, it is difficult to generate classical spreading depression in the rat cerebellar cortex without extensive conditioning by replacing a large fraction of the extracellular Cl⁻ with propionate or benzoate (Nicholson and Kraig 1975; Tobiasz and Nicholson 1982). No conditioning was required to evoke the high-speed propagation in this study, but it was required to evoke spreading depression with microinjection of KCl. Second, ketamine, at the concentrations used for anesthesia in this study, is known to block classical spreading depression (Gorelova et al. 1987; Lauritzen et al. 1988). To evoke classical spreading depression with KCl injection, pentobarbital anesthesia was required. Finally, spreading depression and calcium waves propagate as a wavefront from a central source outward (Newman and Zahs 1997; Yoon et al. 1996). In contrast, the optical signals at the point of initiation increased and remained elevated as the signal spread into the neighboring areas (Fig. 1, C and E–G). These observations demonstrate that the propagating optical response described in this study is not the spreading depression of Leao.

The role of Neutral red in the spread of the optical signal remains unknown. Neutral red has been used as an intracellular vital dye and is noted for its relatively benign characteristics (LaManna and McCracken 1984). Neutral red does not appear to affect the basic electrophysiological properties of the parallel fibers and their postsynaptic targets (Chen et al. 1996, 1998). Still Neutral red may have an unidentified "conditioning" effect on the cerebellar surface that contributed to the spread of acidification.

Previous work found that the pH shifts detected by Neutral red are primarily intracellular (Chen et al. 1998). Several mechanisms may contribute to intracellular acidification. These include metabolic production of CO₂ and lactic acid (Siesjo 1985), GABA channel mediated HCO₃⁻ efflux (Kaila et al. 1990), calcium influx resulting in the release of H⁺ from internal storage sites (Paalasmaa et al. 1994), and glutamate-mediated H⁺ influx (Chen and Chesler 1992). Ion exchangers also may contribute including both the exchangers Cl⁻/HCO₃⁻ (acidifying mechanism) and Na⁺/H⁺ (alkalizing mechanism) known to exist in the rat cerebellar Purkinje cells (Gaillard and Dupont 1990). Surface stimulation and the resultant activation of neuronal activity, including excitatory and inhibitory synaptic connections, are likely to engage several of these mechanisms.

What is the relationship between the pH shift and the suppression of the cerebellar field potentials? Shifts in pH are known to modulate neuronal excitability (Chesler 1990). An increase in...
neuronal acidity depresses voltage-gated sodium channels (Tom-baugh and Somjen 1996), possibly accounting for the suppression of parallel fiber excitability. Decreases in local pH also augment GABA_A channel Cl− conductance (Robello et al. 1994) but attenuate glutamate channel conductance (Traynelis and Cull-Candy 1991), probably contributing to the decreased postsynaptic responsiveness to surface stimulation. Therefore the spread of acidification may contribute directly to the depression in parallel fiber excitability and their postsynaptic targets.

Suppression of synaptic activity outlasted the pH induced optical responses suggesting that other mechanisms mediating cell excitability may be involved (Fig. 2, B and D). One possible mechanism involves an increase of intracellular calcium [Ca^{2+}], induced by activity and acidification (Koch and Barish 1994). Intracellular calcium activates a number of second-messenger cascades in cerebellar neurons that have been associated with long-term depression (for review, see Linden 1994). In Purkinje cells, an increase in [Ca^{2+}] leads to desensitization of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (Crepel and Krupa 1988) and of GABA_A receptor-mediated chloride current (Kano 1996) and facilitates an excitatory amino-acid-dependent postsynaptic anion conductance (Kataoka et al. 1997). All of these mechanisms could contribute to the decreased excitability. Last, nitric oxide produced in response to intracellular calcium activation of a calmodulin-dependent nitric oxide synthase within parallel fibers could diffuse to guanylyl cyclase in Purkinje cells and eventually lead to an inhibition of phosphatases responsible for dephosphorylating AMPA receptor channels in the Purkinje cell membrane (Boxall and Garthwaite 1996; Bredt and Snyder 1989; Ito and Karachot 1992; Lev-Ram et al. 1995; Shibuki and Okada 1991).

The spread of acidification and associated depression in the excitability of the cerebellar circuits may be a neuronal signaling mechanism and/or play a role in pathophysiological processes. The high speed of the spread and relatively long distance traveled provides a mechanism by which neuronal activity in one folium can evoke profound changes in neighboring folia.

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