Modulation of Spreading Depression by Changes in Extracellular pH

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Tong, C. K. and M. Chesler. Modulation of spreading depression by changes in extracellular pH. J Neurophysiol 84: 2449–2457, 2000. Spreading depression (SD) and related phenomena have been implicated in hypoxic-ischemic injury. In such settings, SD occurs in the presence of marked extracellular acidosis. SD itself can also generate changes in extracellular pH (pHo), including a pronounced early alkaline shift. In a hippocampal slice model, we investigated the effect of interstitial acidosis on the generation and propagation of SD in the CA1 stratum radiatum. In addition, a carbonic anhydrase inhibitor (benzolamide) was used to decrease buffering of the alkaline shift to investigate its role in the modulation of SD. pHo was lowered by a decrease in saline HCO3− (from 26 to 13 to 6.5 mM at 5% CO2), or by an increase in the CO2 content (from 5 to 15% in 26 mM HCO3−). Recordings with pH microelectrodes revealed respective pHo values of 7.23 ± 0.13, 6.95 ± 0.10, 6.67 ± 0.09, and 6.97 ± 0.12. The overall effect of acidosis was an increase in the threshold for SD induction, a decrease in velocity, and a shortened SD duration. This inhibition was most pronounced at the lowest pHo (6.5 mM HCO3−) where SD was often blocked. The effects of acidosis were reversible on return to control saline. Benzolamide (10 µM) caused an approximate doubling of the early alkaline shift to an amplitude of 0.3–0.4 U pH. The amplified alkalosis was associated with an increased duration and/or increased velocity of the wave. These effects were most pronounced in acidic media (13 mM HCO3−/5% CO2) where benzolamide increased the SD duration by 55 ± 32%. The initial velocity (including time for induction) and propagation velocity (measured between distal electrodes) were enhanced by 35 ± 25 and 26 ± 16%, respectively. Measurements of [Ca2+]o demonstrated an increase in duration of the Ca2+ transient when the alkaline shift was amplified by benzolamide. The augmentation of SD caused by benzolamide was blocked in media containing the N-methyl-D-aspartate (NMDA) receptor antagonist DL-2-amino-5-phosphonovaleric acid. These data indicate that the induction and propagation of SD is inhibited by a fall in baseline pH characteristic of ischemic conditions and that the early alkaline shift can remove this inhibition by relieving the proton block on NMDA receptors. Under ischemic conditions, the intrinsic alkalosis may therefore enable SD and thereby contribute to NMDA receptor-mediated injury.

INTRODUCTION

Spreading depression (SD) is a transient, propagating electrochemical disturbance in brain associated with rapid movement of ions down their electrochemical gradients. The major extracellular ionic shifts that result from SD include a pronounced elevation of extracellular K+ ([K+]o, etc.), and fall in [Na+]o and [Cl−]o (Kraig and Nicholson 1978). A concurrent influx of Ca2+ results in the decrease of [Ca2+]o by roughly 90% (Nicholson et al. 1977). The associated cellular influx of Ca2+ has been implicated in hypoxic-ischemic brain injury (Balestrino et al. 1989). Thus in animal models of stroke, the occurrence of spontaneous, repetitive SD (Nedergaard and Astrup 1986) has been correlated with the severity of tissue damage. Accordingly, antagonists of the N-methyl-D-aspartate (NMDA) receptor reduce focal infarct size in conjunction with a decrease in the frequency of spontaneous SD (Iijima et al. 1992).

The activity of NMDA receptors is steeply dependent on extracellular H+ and is almost completely inhibited as external pH approaches 6.5 (Tang et al. 1990; Traynelis and Cull-Candy 1990). Since interstitial acidosis falls close to this level in focal ischemia, a substantial block of NMDA receptors by external H+ would be anticipated. Acidosis may therefore be expected to reduce the frequency or impair the propagation of SD. Indeed hypercapnia has been shown to inhibit SD (Gardner-Medwin 1981; Lehenkühler et al. 1973), and hypoxic SD can be delayed by mild acidosis (Tombaugh 1994). On the other hand, the onset of SD is associated with a transient alkalosis (Kraig et al. 1983). Although the mechanism of the alkalosis has not been established, this pH change has a rapid onset and can attain a considerable magnitude during SD. By removing the proton block from NMDA receptors, this alkaline shift may thereby facilitate the generation and propagation of SD.

In this study, we addressed the role of the baseline pHo and the endogenous alkaline shift in the generation and propagation of SD. Extracellular acidification was accomplished by raising the PCO2, or by lowering the bicarbonate concentration of the superfusate. The endogenous alkaline transients were amplified by inhibition of the interstitial carbonic anhydrase. Our results indicate that the occurrence of SD is strongly inhibited by external acidosis; however, once triggered, the duration of SD, the dynamics of calcium entry, and the propagation velocity are markedly influenced by the initial alkaline transient. These effects of the early alkalosis are mediated largely by NMDA receptors. A portion of these results have appeared in preliminary communications (Tong and Chesler 1998, 1999).

METHODS

Preparation and solutions

All procedures were carried out with the approval of the New York University School of Medicine Institutional Animal Care and Use Committee.
Committee. Data were obtained from experiments on 73 hippocampal slices (400 μm thick) prepared from anesthetized, adult, SpragueDawley rats (80–150 g). The slices were maintained in artificial cerebral spinal fluid (ACSF) at room temperature for at least 90 min, then transferred to an interface-style chamber at 35°C for another 30–60 min prior to recording. The ACSF contained (mM) 124 NaCl, 26 NaHCO₃, 3 KCl, 3 CaCl₂, 1.5 MgCl₂, 1 NaH₂PO₄, and 10 glucose; equilibrated with 95% O₂–5% CO₂ to achieve a nominal pH of 7.4. To lower pHₒ, the NaHCO₃ was reduced to 13 or 6.5 mM (with NaCl increased accordingly) to produce an ACSF of pH 7.1 or 6.8, respectively. When measuring changes in [Ca²⁺]ₒ, the ACSF Ca²⁺ was reduced to 1.2 mM. Since the voltage response of an ion selective electrode is proportional to the logarithm of ion activity (Eisenman 1961), the reduction in baseline [Ca²⁺]ₒ served to improve the sensitivity. In experiments using tetramethylammonium (TMA⁺) as a probe of interstitial volume transients, the ACSF included 1 mM TMA chloride.

The carbonic anhydrase inhibitor benzolamide (10 μM; gift of Lederle Laboratories, Pearl River, NY) was added to block interstitial carbonic anhydrase activity and thereby diminish the buffering of rapid alkaline transients (Chen and Chesler 1992a). The resulting effect is the amplification of an interstitial alkalosis mediated by an H⁺ influx (or by an equivalent “proton sink”) (Chen and Chesler 1992b). Blocking interstitial carbonic anhydrase can have the additional effect of inhibiting an alkaline shift when the alkalinosis arises from the efflux of bicarbonate across anion channels of γ-aminobutyric acid (GABA) receptors (Chen and Chesler 1992a; Kaila et al. 1990, 1992). Therefore to prevent confounding effects of a GABAergic bicarbonate efflux and ensure that the carbonic anhydrase inhibitors maximally amplified the SD alkaline shifts, 100 μM picrotoxin (Sigma Chemical) was included in the ACSF. In some experiments, DL-2-amino-5-phosphonovaleric acid (DL-APV; obtained from Tocris Cookson) was added to block NMDA receptors.

**Stimulation and recording**

Construction of double-barreled pH-sensitive microelectrodes (tip diameter of 3–5 μm) and the method of recording and calibrating extracellular pH have been described (Chesler and Chan 1988). pH-sensitive barrels were filled with a proton ionophore cocktail (Fluka Chemical No. 95291) followed by 150 mM NaCl buffered with 50 mM sodium phosphate (pH 7.0). Double-barreled microelectrodes selective for Ca²⁺ or TMA⁺ were constructed similarly, incorporating a Ca²⁺-selective cocktail (Fluka 21048) or TMA⁺-selective cocktail (Corning 477317) in the ion-sensing barrel, followed by 100 mM CaCl₂ or 100 mM TMA⁺, respectively. Reference barrels of all ion selective microelectrodes were filled with 1 M NaCl. Voltages on the ion-sensitive and reference barrels were measured with high-impedance headstages (10¹³ Ω or greater) at unity gain. Reference barrel potentials were continuously subtracted from voltages on the ion barrels. The net ion signal and accompanying reference potentials were monitored on a strip chart recorder and archived to video tape. SD was triggered every 10 min by brief (20–200 ms) ejection of 1.2 M KCl into the CA1 stratum radiatum via a micropipette (tip, approximately 5 μm) connected to a Picospirtzer (General Valve). The KCl pulse duration was not changed during successive SDs except during a few experiments in low pH Ringer where the threshold pulse for SD initiation increased (see Results). Propagation of SD toward the CA3 region was monitored by a double-barreled ion-selective microelectrode placed approximately 400 μm from the KCl injection pipette (in CA1 s. radiatum), allowing a simultaneous recording of extracellular potential (Vₑ) and either pHₒ, [Ca²⁺]ₒ, or [TMA⁺]ₒ. A single-barreled micropipette monitored extracellular potential (Vₑ) closer to the CA3 region at a distance of approximately 800 μm from the ejection site (Fig. 1A). For each experiment, distances between the electrodes were measured with an accuracy of ±5%. While the electrodes were vertically lowered, the Schaffer collateral fibers were stimulated at roughly 0.1 Hz, and the evoked field excitatory post synaptic potential was monitored. Electrodes were positioned where the maximum field potential amplitude was noted, which corresponded to a recording depth of 150–250 μm. Once placed, the electrodes were not moved for the duration of the SD experiment.

**Definition of parameters**

The onset of SD was defined as the point of inflection on Vₑ or V₂ that commenced the rapid negative phase of the DC shift. When recording pHₒ or [Ca²⁺]ₒ this inflection always coincided with the sudden extracellular alkaline shift or fall in [Ca²⁺]ₒ (Fig. 1B). The recovery phase of the SD voltage trace did not have a similar recognizable transition point. Therefore the SD “duration” was defined as the interval between the time of half-maximal voltage shift during onset and recovery of the DC field potential. The SD delay was defined as the time interval from the instant of KCl injection until the onset of SD at V₁. Because of variability in the distance between the KCl injection pipette and the V₁ electrode, the measured distances were divided by the delays, providing a measure of “initial velocity” when averaging data from several slices. This initial velocity was therefore dependent on the time required for initiation of SD around the injection pipette as well as the time for propagation to the V₁ electrode. The SD “propagation velocity” was calculated from the time interval between onset of SD at V₁ and V₂ (defined as conduction time) and from the measured V₁ – V₂ electrode spacing. These parameters are displayed in Fig. 1C.

**Data analysis and response stability**

Only those slices in which SD could be repeatedly evoked under control conditions were included in the analysis. To obtain data from a given slice, parameters were averaged from two to three sequential SD waves evoked under control conditions, and two to three evoked after a change to a test solution (e.g., benzolamide or low pH). Statistics compiled for several slices were expressed as means with standard deviation. Paired trials refer to measurements made on the same slice (e.g., before and after addition of benzolamide). Comparisons were made with a paired t-test, unless indicated. Values of n refer to the number of slices.

The analysis of the effect of benzolamide on the SD parameters depended on the stability of the successive SD responses. Most experiments were conducted in the presence of picrotoxin with or without added APV. The major impact of picrotoxin (100 μM) on the control SD responses was an increase in the propagation velocity and duration. Picrotoxin was typically included from the start of an experiment; however, in four experiments (in 13 mM HCO₃⁻) in which it was added later, we noted an increase in propagation velocity and duration of 57 ± 29 and 44 ± 39%, respectively. In the presence of picrotoxin, the principal effect of APV (50 μM) consisted of a decrease in the mean duration (e.g., from a mean of 13.9 ± 3.4 s in 13 slices, to 8.6 ± 1.5 s in 5 slices, in 13 mM HCO₃⁻) and a decrease in the initial velocity (e.g., from a mean of 8.7 ± 3.1 mm/min in 16 slices vs. 6.2 ± 1.8 mm/min in 9 slices), similar to the in vivo actions of NMDA receptor antagonists on SD (Marrannes et al. 1988). Recordings were not performed until the variability in these parameters was minimal. Typically, the first five to eight SD trials (during the initial 50–80 min) displayed a progressively increasing velocity and duration and therefore were not used in the analysis. After this period, SD became more consistent; however, either small increases or decreases in these parameters could occur between successive trials. Among the SD responses that were analyzed before and after addition of benzolamide, there was a modest increase in the velocity and duration. For example, among the responses analyzed in 13 mM HCO₃⁻ ACSF with picrotoxin, the average change in initial velocity between responses amounted to 2 ± 6%. The corresponding average
change in duration was 5 ± 3% (n = 17). The possible impact of these variations are considered in the DISCUSSION.

RESULTS

Effect of elevated PCO₂ on SD

In six slices bathed in picrotoxin, the external pH was lowered by elevation of the PCO₂ at constant bicarbonate concentration. The induction of SD was tested sequentially in control ACSF (5% CO₂-95% O₂, 26 mM HCO₃⁻, pH 7.4), high CO₂ solution (15% CO₂-85% O₂, 26 mM HCO₃⁻, pH ~ 6.92), and again in control media. Solutions were superfused for at least 30 min prior to the induction of SD. In the high CO₂ media, the interstitial pH was 6.97 ± 0.12 in four slices. Elevated CO₂ reversibly diminished the ability of the tissue to generate SD. This was evident at V₁ as either an outright block of SD (1 slice), an increase in the required KCl pulse duration (1 slice), and/or a significant decrease in the initial velocity. In five slices where SD was initiated, the initial velocity fell, and in two slices, SD induction required an increase in KCl pulse duration; however, the SD wave failed to propagate to V₂. In the total of nine slices studied with and without picrotoxin, the effects of elevated CO₂ were reversible after return to normal ACSF (Fig. 2).

Effect of low-bicarbonate ACSF

In eight slices bathed in picrotoxin, external pH was lowered via a reduction in the ACSF bicarbonate concentration. The effect on SD was tested by sequentially switching from 26 mM bicarbonate ACSF (pH 7.4) to 13 mM bicarbonate fluid (pH 7.1), then to 6.5 mM bicarbonate media (pH 6.8), followed by a return to control solution. After an equilibration time of at least 30 min in each solution, the induction of SD was attempted with a given KCl pulse. The solutions of pH 7.4, 7.1, and 6.8 resulted in a corresponding pH₀ of 7.23 ± 0.13, 6.95 ± 0.10, and 6.67 ± 0.09, respectively.

In control solution (26 mM bicarbonate), a propagating SD could be readily induced in all slices. Sequential transition from 13 to 6.5 mM bicarbonate caused a stepwise decrease in the ability of the slice to generate a SD or sustain a propagating wave. These effects were completely reversed on return to control ACSF (Figs. 3 and 4).

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After transition to 13 mM bicarbonate, an SD could usually be elicited with the same KCl pulse. In two slices, however, the SD was blocked (Fig. 4, A and B) but could be induced after...
doubling the duration of the KCl pulse (Fig. 4C). Although SD could be consistently elicited in 13 mM bicarbonate, this solution had marked effects on the propagation of the wave. Initial velocity fell from 8.3 ± 3.2 to 6.3 ± 2.2 mm/min (n = 8, P < 0.05). Moreover, in three of eight slices, the SD did not propagate to V2. In cases where an SD reached V2, the propagation velocity fell from 6.0 ± 1.3 to 4.8 ± 1.4 mm/min (n = 5, P < 0.05).

Transition to 6.5 mM bicarbonate media had more pronounced effects on the SD wave. In three slices, SD could not be induced, despite increases in the KCl pulse duration (Fig. 4). In the remaining five slices, the initial velocity fell from 10.0 ± 2.6 to 4.8 ± 1.5 mm/min with a decrease in SD duration from 21.7 ± 4.6 to 11.2 ± 4.0 s. Among these five slices, the SD propagated to V2 in only three cases with an average propagation velocity of only 3.8 ± 1.2 mm/min and a decrease in the amplitude of the negative voltage shift to 62 ± 18% of control.

The effect of low-bicarbonate media was studied largely in the presence of picrotoxin in order that results be comparable with studies of carbonic anhydrase inhibition, described in the
following text. In experiments performed in the absence of picrotoxin, similar reversible effects were noted in three slices for which the media was switched between ACSF containing 26, 13, and 6.5 mM bicarbonate. In two slices, the SD did not propagate to $V_2$ in 13 mM bicarbonate. In all three slices, the SD was completely blocked in 6.5 mM bicarbonate. Full recovery was noted after return to control ACSF.

**Effect of benzolamide on SD in normal ACSF**

Benzolamide is a potent, charged (pKa $= 3.2$) inhibitor of carbonic anhydrase that crosses cell membranes poorly (Travis et al. 1964). When superfused in vitro (Chen and Chesler 1992a; Kaila et al. 1992) or in vivo (Huang et al. 1995), this agent causes rapid inhibition of interstitial carbonic anhydrase activity. The principal effect on activity-dependent pH$_3$ shifts is the immediate amplification of early alkaline transients, which has been explained by the reduced rate of H$^+$ buffering when CO$_2$ hydration becomes uncatalyzed (Chen and Chesler 1992b).

In normal ACSF (pH 7.4), superfusion of 10 $\mu$M benzolamide had a pronounced effect on the SD alkaline shift, increasing the amplitude from 0.15 ± 0.09 to 0.36 ± 0.28 unit pH ($n = 7$, $P < 0.05$). There was no significant effect on the late acidosis. This amplification of the alkaline pH$_3$ transient was associated with a small, but significant prolongation of the SD. The duration at the distal recording site ($V_2$) increased from a mean of 12.5 ± 4.8 to 14.8 ± 5.6 s ($n = 6, P < 0.01$). A significant prolongation was also noted at the proximal recording site ($V_1$) with an increase from 16.6 ± 4.4 to 19.1 ± 5.5 s ($n = 7, P < 0.01$). Benzolamide did not significantly affect the initial velocity or the propagation velocity in normal ACSF.

**Effect of benzolamide on SD in acidic ACSF**

In ACSF containing 13 mM bicarbonate (pH 7.1, pH$_o$ 6.8–7.0), benzolamide amplified the alkaline shift from 0.20 ± 0.07 to 0.38 ± 0.17 U pH ($n = 16$), whereas the associated late acidosis was unchanged. Compared with normal ACSF, the duration of the SD was more markedly increased by benzolamide in the acid media (Fig. 5). At the proximal recording site ($V_1$), the SD duration increased by 26 ± 21%, from 13.9 ± 3.4 to 17.4 ± 4.7 s ($n = 13, P < 0.001$). At the distal recording site ($V_2$), the duration increased by 55 ± 32%, from 9.7 ± 3.1 to 14.3 ± 2.8 s ($n = 9, P < 0.001$).

Whereas benzolamide did not affect the speed of SD in normal media, a clear increase in velocity was noted in ACSF containing 13 mM bicarbonate. The initial velocity increased from 8.7 ± 3.1 to 11.6 ± 3.9 mm/min (by 35 ± 25%, $n = 16, P < 0.001$). The propagation velocity increased from 5.0 ± 1.4 to 6.4 ± 2.0 mm/min (26 ± 16%, $n = 12, P < 0.001$).

**Involvement of NMDA receptors in the augmentation of SD**

To test whether NMDA receptors were involved in benzolamide-induced augmentation of SD, similar experiments were carried out in acidic ACSF (13 mM bicarbonate, pH 7.1) containing the NMDA antagonist APV (50 $\mu$M). In the presence of APV, superfusion of benzolamide amplified the alkaline shift from 0.23 ± 0.08 to 0.41 ± 0.13 U pH ($n = 10$). This amplification was not significantly different from that in the absence of APV. With APV present, the duration of SD was not significantly affected by benzolamide despite the marked increase in the alkalosis (Fig. 6). At $V_1$, the durations were 8.6 ± 1.5 versus 9.5 ± 0.8 s before and after addition of benzolamide, respectively (increase of 12 ± 14%, $n = 5, P > 0.1$). At $V_2$, the respective durations were 9.6 ± 2.7 versus 10.3 ± 2.4 s (increase of 8 ± 10%, $n = 6, P > 0.05$). In the presence of APV, the speed of SD was similarly unaffected by benzolamide. The mean initial velocity was 6.2 ± 1.8 mm/min prior and 6.9 ± 2.9 mm/min following addition of benzolamide (increase of 11 ± 21%, $n = 9, P > 0.1$). The respective propagation velocities were 5.3 ± 1.5 versus 5.8 ± 1.3 mm/min (increase of 13 ± 14%, $n = 8, P > 0.05$).

![Diagram](http://jn.physiology.org/Downloadedfrom/10.220.32.2)
Effect of benzolamide on \([Ca^{2+}]_o\) transients during SD

Since the augmentation of SD by benzolamide was mediated largely by NMDA receptors, a concomitant modulation of \(Ca^{2+}\) influx and change in the \([Ca^{2+}]_o\) transient could be anticipated. This was tested in acidic ACSF (13 mM bicarbonate, pH 7.1), where the effect of benzolamide was most pronounced. Prior to addition of benzolamide, SD was associated with a mean fall of \([Ca^{2+}]_o\) of 1.09 ± 0.03 mM (n = 5). Although most of the interstitial \(Ca^{2+}\) entered cells during the SD, the addition of benzolamide caused a small but consistent increase in the transient (Fig. 7) which averaged 1.12 ± 0.02 mM (n = 5, P < 0.05). Since the sensitivity of the \(Ca^{2+}\)-selective microelectrode increases as \([Ca^{2+}]_o\) falls, this difference (30 ± 22 μM), although small, was readily detectable, represented by a disparity in the voltage responses of the \(Ca^{2+}\) electrodes that averaged 4.2 ± 3.4 mV. Benzolamide caused a similar augmentation in the presence of 50 μM APV, indicating that the increase

![Diagram of Control and Benzolamide effects on pH and voltage](image1)

**FIG. 5.** Effect of benzolamide on SD. Superfusion of 10 μM benzolamide amplified the SD alkaline shift and markedly increased the SD duration. The SD delay and conduction time were shortened compared with control intervals (● and □, respectively) indicating increased initial velocity and propagation velocity. Solution contained 13 mM bicarbonate (5% CO₂) and 100 μM picrotoxin.

**FIG. 6.** Effect of benzolamide is blocked by DL-2-amino-5-phosphonovaleric acid (DL-APV). In the presence of 50 μM APV, the SD alkaline shift was still amplified by 10 μM benzolamide; however, the duration, delay, and conduction time did not change significantly. Solution contained 13 mM bicarbonate (5% CO₂) and 100 μM picrotoxin.
in the [Ca\(^{2+}\)]\(_o\) transient was independent of NMDA receptors. In APV, the [Ca\(^{2+}\)]\(_o\) transient averaged 1.06 ± 0.03 mM before and 1.08 ± 0.03 mM after addition of benzolamide \((n = 5, P < 0.01)\).

Benzolamide had a pronounced effect on the time course of the Ca\(^{2+}\) decrease in conjunction with the prolongation of the SD wave (Fig. 7). After addition of benzolamide, the transient fall in [Ca\(^{2+}\)]\(_o\) increased in duration from 13.7 ± 5.7 to 24.0 ± 9.8 s \((n = 5, P < 0.01)\), representing a mean prolongation of 78 ± 23%. In the presence of APV, the effect of benzolamide on the [Ca\(^{2+}\)]\(_o\) transient was markedly diminished (not shown), with a prolongation of 18 ± 10% \((from 13.0 ± 1.3 to 15.4 ± 2.6 s, n = 5, P < 0.02)\). The effect of benzolamide in the presence of APV was significant but was far less than in its absence \((18 ± 10 vs. 78 ± 23\%\), respectively, \(P < 0.001\) unpaired \(t\)-test).

The remaining, APV-insensitive augmentation of SD could be mediated by a high-threshold, voltage-gated Ca\(^{2+}\) conductance as these Ca\(^{2+}\) channels can display a pronounced sensitivity to extracellular pH (Tombaugh and Somjen 1998). To test one such channel, the effect of benzolamide was studied in the presence of APV plus the L-type antagonist nimodipine \((10 \mu M)\). In this solution, benzolamide still prolonged the [Ca\(^{2+}\)]\(_o\) transient \((by 22 ± 10\%, from 9.6 ± 2.2 to 11.6 ± 2.0 s, n = 5, P < 0.01)\). This augmentation was not different from that seen in the presence of APV alone \((P > 0.1, \text{unpaired} \ t\text{-test})\), suggesting that the APV-insensitive augmentation was not mediated by L-type calcium channels.

**Effect of benzolamide on extracellular volume changes during SD**

The magnitude and duration of interstitial ionic shifts are partially governed by activity-dependent changes in the extracellular volume fraction (Dietzel et al. 1980). To determine whether benzolamide affected the extracellular volume changes associated with SD, we measured the changes in [TMA\(^{+}\)]\(_o\). In ACSF containing 13 mM bicarbonate \((pH 7.1\) with 1 mM TMA\(^{+}\)), SD was associated with an increase in [TMA\(^{+}\)]\(_o\), corresponding to shrinkage of the interstitial compartment to 42 ± 4% of its original volume \((n = 5)\). Following addition of benzolamide, the peak volume change of the extracellular space was not significantly different, shrinking to 45 ± 4% of control (Fig. 8). The duration of the volume transient was significantly increased by benzolamide (by 30 ± 24%)

**FIG. 7.** Effect of benzolamide on SD-induced [Ca\(^{2+}\)]\(_o\) shift. Superfusion of 10 \(\mu M\) benzolamide caused a small, but significant increase in the [Ca\(^{2+}\)]\(_o\) shift and a marked delay in the [Ca\(^{2+}\)]\(_o\) recovery. - - - , superimposed control [Ca\(^{2+}\)]\(_o\) shift. Solution contained 13 mM bicarbonate \((5\% CO_2)\) and 100 \(\mu M\) picrotoxin.

**FIG. 8.** Benzolamide does not increase extracellular volume transient during SD. Amplitude of the [TMA\(^{+}\)]\(_o\) transient during SD was not significantly changed in the presence of 10 \(\mu M\) benzolamide. The duration of the [TMA\(^{+}\)]\(_o\) transient increased along with the prolongation of the SD. Solution contained 1 mM TMA\(^{+}\), 13 mM bicarbonate \((5\% CO_2)\), and 100 \(\mu M\) picrotoxin.
DISCUSSION

SD and SD-like phenomena are important factors in the manifestation of hypoxic-ischemic brain injury (Balestrino et al. 1989; Iijima et al. 1992; Nedergaard and Astrup 1986). In these pathological settings, SD can be expected to occur in an extracellular microenvironment of low pH (Kraig et al. 1985; Nedergaard et al. 1991; von Hanxwehr et al. 1986). The lactic acidosis that occurs in the absence of oxygen will be characterized by a fall in the interstitial bicarbonate and a rise in the tissue PCO₂ (Hansen 1985). We have therefore examined SD under both conditions. In addition to the effect of baseline pH on SD, endogenous pH transients that develop during the generation of SD may also be expected to influence the phenomenon. The alkaline shift at the onset of SD is of particular interest since it occurs with a large eflux of glutamate (Fabricius et al. 1993; Van Harreveld and Fifkova 1970) and may therefore remove the proton block from NMDA receptors at the moment agonist levels rise.

Whether achieved by lowering bicarbonate, or raising the PCO₂, a decrease in the baseline pH hindered the generation and propagation of SD. The interstitial pH fell to comparable levels in 15% CO₂ and 13 mM bicarbonate ACSF, and both media had a similar mild inhibitory effect on SD. Each solution would also be expected to lower intracellular pH, although the elevation of CO₂ may be expected to cause the greater cytoplasmic acidification due to the high membrane permeability of this gas (Roos and Boron 1981). However, without direct intracellular measurements, it remains uncertain how cytoplasmic acidosis affects SD.

The most obvious effect of low pH media was a rise in the threshold for SD induction. This was most evident when the initiation of SD required an increase in the duration of the KCl pulse. However, the slowing of the initial velocity also reflected an inhibitory effect on the generation of SD since the delay between stimulus (KCl) and the appearance of SD at V₁ entailed time required for both induction and propagation.

The propagation velocity, determined by the V₁-V₂ conduction time, was also diminished at low external pH. The most pronounced effects were noted when ACSF bicarbonate was lowered to 6.5 mM (which correlated with a mean interstitial pH of 6.67). Under these conditions, SD could be induced and recorded at V₁, but further propagation to V₂ was often aborted.

These findings are consistent with the delay in hypoxic SD that was noted when hippocampal slices were bathed in mildly acidic solutions (Tombaugh 1994). In chick retina, by contrast, similar changes in SD conduction required large deviations in fluid pH. Decreases in pH to 6.4 and increases to 9.4 produced a respective fall and rise in SD velocity; however, retinal SD was unaltered by solution changes less than 0.5 U pH (Skelton et al. 1983).

Deceleration of SD induced by low pH might be expected to prolong the event, as the wave would dwell in a locale for a longer period of time. Instead the slower SD was associated with a decrease in the SD duration. Both the slowing and the shortening of SD might be explained by an increased proton block of the NMDA receptor. Loss of NMDA receptor activation would be consistent with an increased threshold for SD and loss of longer duration components of the wave. This interpretation is supported by the shorter duration and decreased initial velocity of SD observed in APV-containing media (see METHODS).

Experiments with benzolamide supported the notion that the pH sensitivity of SD is mediated via NMDA receptors. This carbonic anhydrase inhibitor caused a large amplification of the alkaline transient at the onset of SD and concomitant increases in duration and velocity of the wave. These effects were most pronounced in acidic media where the rapid alkalosis would be expected to transiently remove a sizable proton block from NMDA receptors. Indeed the facilitation of the SD was largely eliminated when experiments were conducted in the presence of the NMDA receptor antagonist APV.

The effect of benzolamide could be overestimated if the SD responses increased between successive trials. A uniform drift was not evident, as either a small increase or decrease in parameters could occur between two given trials. Yet overall, there was a small average increase in the velocity and duration among the analyzed SD responses. Subsequent to adding benzolamide, these changes could have comprised a fraction of the apparent effect. For instance, given a mean increase in SD duration and initial velocity of 55 and 35%, respectively, we estimate that one fifth of these changes could have been due to an ongoing prolongation of the responses. Most of the increases, however, were attributable to the effect of benzolamide.

These results compliment studies of synaptic physiology in which benzolamide caused long term enhancement of orthodromic field potentials (Taira et al. 1993) and a prolongation of NMDA receptor-mediated synaptic currents (Gottfried and Chesler 1994). A similar enhancement of synaptic currents preceding the SD wave may have contributed to the increase in propagation velocity noted in this study.

A consequence of enhanced NMDA receptor activity might be increased entry of calcium ions. This did not manifest as a far greater fall in [Ca²⁺], because most of the interstitial Ca²⁺ left the extracellular compartment during SD whether or not benzolamide was added to enhance the alkaline transient. Instead, benzolamide caused a more sustained fall in external Ca²⁺. This was not due to differential changes in the extracellular volume fraction as the amplitude of the TMA⁺ signals was unaffected by benzolamide. Rather the behavior of both Ca²⁺ and TMA⁺ was consistent with the overall prolongation of the SD wave caused by amplification of the alkaline transient.

The mechanism of the alkaline shift has not been firmly established. Because the response can be markedly reduced in the absence of Ca²⁺, it has been suggested that the Ca²⁺-H⁺ exchange property of the plasmaemal Ca²⁺-ATPase may be responsible for all or part of the pH change (Paalasmaa et al. 1994; Smith et al. 1994). Such a link to Ca²⁺ entry would suggest additional interactions between pH shifts and Ca²⁺ dynamics during SD.

The present results suggest that the behavior of interstitial pH can have an important bearing on the pathophysiology of brain ischemia. Indeed, given an extracellular pH as low as 6.6 in focal ischemia (Nedergaard et al. 1991), the contribution of NMDA receptors to excitotoxic injury might occur by virtue of the alkalosis at the onset of SD. Uncertainties regarding extrapolation of these results to the pathological context should
be considered, however. For instance, although the occurrence of prolonged SD has been noted in focal ischemia (Nedergaard and Astrup 1986), the associated pH dynamics are not known. Moreover the activation of interstitial carbonic anhydride (the foremost factor governing the amplitude of extracellular alkaline transients) is uncertain in ischemic brain.

Uncertainty also remains concerning the role of GABA<sub>A</sub> receptors. Bicarbonate efflux across these anion channels can cause an extracellular alkalosis that is inhibited by carbonic anhydrase inhibitors (Chesler and Kaila 1992). If an evoked alkaline shift contained such a component, the amplification by benzolamide would not be maximal (Taira et al. 1995). Indeed in a few instances in which benzolamide was used without picrotoxin, the increase in the SD alkaline shift was slight (unpublished observations). Therefore to ensure the maximum amplification of the alkalosis, we routinely included picrotoxin in the ACSF. The role of GABA<sub>A</sub> responses by extracellular pH can be either positive or negative and is likely to depend on subunit composition of particular GABA receptors (Traynelis 1998).

In summary, the present data demonstrate that decreases in interstitial pH, typical of ischemic brain, have a marked inhibitory effect on the induction, duration, and propagation of SD. The transient alkalization at the onset of SD can overcome this hindrance by removing the proton block from NMDA receptors. These results suggest that the link between SD and NMDA receptor-mediated injury can be strongly influenced by the accompanying endogenous shifts in extracellular pH.

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