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*Rapid Rise of Extracellular pH Evoked by Neural Activity is*
*Generated by the Plasma Membrane Calcium ATPase*

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Surface pH Shifts on CA1 Pyramidal Neurons

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Abstract

In hippocampus, synchronous activation of CA1 pyramidal neurons causes a rapid, extracellular, population alkaline transient (PAT). It has been suggested that the plasma membrane Ca\(^{2+}\)-ATPase (PMCA) is the source of this alkalinization, as it exchanges cytosolic Ca\(^{2+}\) for external H\(^+\). Evidence supporting this hypothesis, however, has thus far been inconclusive. We addressed this long-standing problem by measuring surface alkaline transients (SATs) from voltage clamped CA1 pyramidal neurons in juvenile mouse hippocampal slices, using concentric (high-speed, low-noise) pH microelectrodes placed against the somata. In saline containing benzolamide (a poorly-permeant carbonic anhydrase blocker), a 2 s step from -60 to 0 mV caused a mean SAT of 0.02 unit pH. Addition of 5 mM HEPES to the ACSF diminished the SAT by 91 percent. Nifedipine reduced the SAT by 53 percent. Removal of Ca\(^{2+}\) from the saline abolished the SAT, and addition of BAPTA to the patch pipette reduced it by 79 percent. The inclusion of carboxyeosin (a PMCA inhibitor) in the pipette abolished the SAT, whether it was induced by a depolarizing step, or by simulated, repetitive, antidromic firing. The peak amplitude of the “antidromic” SAT of a single cell averaged 11 percent of the PAT elicited by comparable real antidromic activation of the CA1 neuronal population. Caloxin 2A1, an extracellular PMCA peptide-inhibitor, blocked both the SAT and PAT by 42 percent. These results provide the first direct evidence that the PMCA can explain the extracellular alkaline shift elicited by synchronous firing.
Introduction

During synchronous neural activity, a rapid rise in extracellular pH (pHe) occurs in the hippocampus, cerebellum and some cortical regions. The hippocampal alkaline transient can attain magnitudes as large as 0.1 - 0.2 unit pH (Chesler, 1990; 2003) and can be detected within tens of ms following a stimulus (Gottfried and Chesler, 1996; Tong et al., 2006). The alkalosis was amplified by inhibitors of carbonic anhydrase, both in vivo (Kraig et al., 1983) and in vitro (Walz, 1989; Chen and Chesler, 1992a). This effect was caused by decreased extracellular buffering, due to the slower rate of CO$_2$ hydration. Thus, the alkaline response was attributed mainly to the rapid removal of protons from the extracellular space, and was termed a “proton sink” (Chesler and Kaila, 1992; Chesler, 2003).

The cause of the proton sink has not been determined. The amplification caused by carbonic anhydrase inhibitors distinguished it from other mechanisms of activity dependent alkalinization. For example, an alkalosis can occur due to the efflux of HCO$_3$- across GABA-A channels (Kaila and Voipio, 1987), however such responses are inhibited by carbonic anhydrase blockers (Kaila et al., 1992). Similarly, a slower alkaline shift in the caudate nucleus that was correlated with increases in blood flow (and presumed washout of CO$_2$) was also reduced by block of carbonic anhydrase (Venton et al., 2003).

It has been proposed that the proton sink arises from the neuronal plasma membrane Ca$^{2+}$-ATPase (PMCA) (Schwiening et al., 1993), a ubiquitous transporter that
exchanges internal Ca\textsuperscript{2+} for external H\textsuperscript{+} (Carafoli, 1991; Carafoli and Stauffer, 1994). To date, the evidence supporting this long-standing hypothesis has been inconclusive. For example, the alkaline response depends on the presence of external Ca\textsuperscript{2+} (Grichtchenko and Chesler, 1996; Paalasmaa and Kaila, 1996), but myriad processes can be influenced by Ca\textsuperscript{2+} influx. Additionally, the entry of Ca\textsuperscript{2+} is typically associated with cytosolic acidification (Meech and Thomas 1977; Irwin et al., 1994; Wang et al., 1994; Trapp et al., 1996), which is in keeping with the operation of the PMCA. However, Ca\textsuperscript{2+} entry can acidify cells by means other than a proton influx across the plasma membrane. Generation of metabolic acid, as well as Ca\textsuperscript{2+}-H\textsuperscript{+} exchange across the membranes of the endoplasmic reticulum (ER) or mitochondria, could also cause an intracellular acidification (Chesler, 2003). Pharmacologic dissection of this acidosis is not possible, since both the PMCA and these organellar transporters are blocked by the commonly employed inhibitors such as orthovanadate and eosin (O’Neal et al., 1979; Kosterin et al., 1996; Watson et al., 2003).

Thus, the suppression of a Ca\textsuperscript{2+}-dependent fall in intracellular pH by these agents (Trapp et al., 1996) cannot necessarily be attributed to block of the PMCA.

To avoid such ambiguity, a PMCA-related proton influx might be detected via the rise in pH at the extracellular surface of a single neuron. Proof of principle was first demonstrated by Schwiening et al. (1993) on giant snail neurons, and later in studies of large, isolated retinal neurons from the skate (Molina et al., 2004) and catfish (Kreitzer et al., 2007). Similar experiments have not been performed on single mammalian neurons.
To relate such surface measurements to the extracellular proton sink generated by synchronous neural activity, these experiments would have to be performed in situ.

Previously, such efforts would have been hampered by reliance on conventional pH microelectrodes, which have high inherent noise, and poor temporal resolution. Here, we address this issue in mouse hippocampal slices, using recently designed, high speed, low noise, concentric pH microelectrodes (Fedirko et al., 2006). By placing the pH electrode against the soma of a voltage clamped CA1 pyramidal neuron, we demonstrate rapid surface alkaline shifts elicited by depolarization. This alkalinization was apparent within 200 ms, required entry of Ca$^{2+}$, and was inhibited by intracellular and extracellular PMCA inhibitors. The surface response of a singly activated neuron amounted to 11 percent of the peak alkalosis attained by antidromic activation of the pyramidal neuron population. Moreover, caloxin, an extracellular peptide inhibitor of the PMCA, curtailed the surface and population responses to the same degree. These data provide strong evidence that the extracellular alkaline shift evoked by synchronous activity is generated by the neuronal PMCA. A preliminary report has appeared (Makani and Chesler, 2008).
Materials and Methods

Preparation and Solutions.

Brain slice preparation: Transverse hippocampal slices were prepared from P6 – P14 mice of either sex. All procedures were performed with the approval of the New York University Langone School of Medicine Institutional Animal Care and Use Committee. The brain was blocked in ice-cold artificial cerebrospinal fluid (ACSF) and cut into 250 µm slices using a Vibratome. The slices were incubated in standard ACSF at room temperature for at least 1 hour before use. Experiments were then conducted in a submersion-style incubation chamber at 32°C. The standard ACSF contained (in mM): 124 NaCl, 3.0 KCl, 1.0 CaCl₂, 1.5 MgCl₂, 26 NaHCO₃, 1.0 NaH₂PO₄, and 10 D-glucose, with pH 7.4 (equilibrated with 95 percent O₂ and 5 percent CO₂). In some experiments, we utilized a modified ACSF that contained 1, 5, or 20 mM HEPES acid in addition to 26 mM HCO₃⁻. The HEPES solutions were formulated using an appropriate addition of extra NaHCO₃, so as to maintain a pH of 7.4 in 5 percent CO₂, and therefore a final HCO₃⁻ concentration of 26 mM (Stewart, 1978). To maintain a constant Na⁺ concentration, the NaCl content was reduced accordingly. In several experiments, a zero Ca²⁺ solution was made by omitting CaCl₂ from the Ringer and adding 1 mM EGTA. Where Cd²⁺ was added, phosphate was removed from the saline to prevent precipitation. The poorly permeant carbonic anhydrase inhibitor benzolamide (10 µM) was included (unless otherwise noted) in order to decrease the effective extracellular buffering capacity, thereby keeping all extracellular pH changes relatively large, and independent of possible
differences in extracellular carbonic anhydrase activity (Chen and Chesler, 1992a). To prevent \( \text{HCO}_3^- \) efflux through GABA-A anion channels, which could cause a confounding extracellular alkalosis (Kaila and Voipio, 1987; Chen and Chesler, 1992b), picrotoxin was included in all experiments.

Drugs.

Drugs were added to the external ACSF, or to the intracellular pipette solution, as noted, in the following concentrations: DL-2-Amino-5-phosphonovalerate (APV; 50 \( \mu \text{M} \)), 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX; 10 \( \mu \text{M} \)) benzolamide (10 \( \mu \text{M} \)), picrotoxin (100 \( \mu \text{M} \)), lidocaine N-ethyl bromide (QX-314; 4 mM), cadmium (300 \( \mu \text{M} \)), nifedipine (25 \( \mu \text{M} \)), carboxyeosin (0.5 or 5 \( \mu \text{M} \)), and BAPTA (1 mM). Benzolamide was a gift from Dr. Erik Swenson (University of Washington, Seattle, WA). Caloxin 2A1 (VSNSNWPSFPSSGGG-NH₂) was custom-synthesized by Bio Basic Inc. (Ontario, Canada). All other agents were obtained from Sigma-Aldrich (St. Louis, MO).

Concentric pH-Sensitive Microelectrodes.

pH-sensitive microelectrodes with response times of a few ms. were fabricated as detailed by Fedirko and colleagues (Fedirko et al., 2006). In brief, a thin-walled borosilicate glass capillary with outer diameter (OD) of 2.0 mm, and inner diameter (ID) of 1.5 mm (AM Systems 6185, Carlsborg, WA) was pulled to a tip size of 2–4 \( \mu \text{m} \), and then silanized by injection of pure \( N, N \)-dimethyltrimethylsilylamine (Fluka 41720), followed by heating with a hot air gun. A pH-selective mixture (Fluka 95291) was then introduced into the tip
by suction. The inner micropipette was pulled from thin-walled glass (OD 1.2 mm, ID 0.9 mm; #6160; A-M Systems) to a tip diameter of approximately 0.5 µm. This pipette was filled with phosphate-buffered 3 M KCl (pH 7.4) and inserted within the outer, ion-selective barrel and into the ion exchange column. With its tip 4 – 6 µm from the end of the outer pipette, the inner pipette was secured in place with dental wax. The pH microelectrodes were calibrated in 50 mM K+/Na+ phosphate buffers of pH 6.87 and 7.42. The slope response for the concentric electrodes was 57-59 mV per decade change in H+ activity. The mean response time constant of similar concentric pH microelectrodes constructed in this laboratory was reported to be 15 ms. (Fedirko et al., 2006).

**Whole Cell Recording.**

The somata of CA1 pyramidal neurons were visualized under infrared differential interference contrast microscopy using a Zeiss Axioskop 2 Plus, fixed-stage microscope, fitted with a 40x, water immersion objective (0.75 numerical aperture), and an Olympus Optical 150 video camera. Patch pipettes were pulled from 1.5 mm OD x 1.12 mm ID borosilicate tubing (World Precision Instruments, Sarasota, FL) using a Narishige PP-830 two-stage puller (Japan). The intracellular filling solution contained the following (in mM): 120 K-gluconate, 20 KCl, 2.0 MgCl2, 25 Na-HEPES, and 2 Mg2+-ATP. In a few experiments, K-gluconate was replaced with Cs-gluconate and QX-314 was added to the filling solution. After adjusting the pH to 7.3 with KOH (or CsOH), the final osmolarity was 280-290 mOsm. Pipettes had resistances of 3 – 5 mΩ. In experiments in which carboxyeosin or BAPTA was added to the intracellular solution, pipette tips were first
dipped in drug-free solution, then back-filled with the drug-containing solution. Data were acquired using an Axopatch 1D amplifier and Digidata board 1320A, controlled by Clampex 8.2 and analyzed using ClampFit (Molecular Devices, Union City, CA). Data were accepted if cells had a series resistance of less than 25 M\(\Omega\) that did not change by more than 20 percent during the experiment.

Surface Alkaline Transients Elicited by Voltage Clamp Steps.

Prior to placement of the surface pH microelectrode, a gigohm seal was obtained on the targeted soma with a standard patch pipette. The concentric pH microelectrode was then advanced until its tip made contact with the same cell body. Brief suction was then applied to the patch pipette to break through into the whole-cell configuration. Surface pH responses were elicited by depolarizing voltage clamp steps from a holding potential of -60 mV. The depth of targeted cells ranged from the slice surface to approximately 100 \(\mu\)m. The depth did not correlate with the size of surface pH responses. In these single cell experiments, the concentric pH electrode was used without an accompanying reference pipette. To determine the degree to which DC artifacts contaminated the surface pH records, separate experiments were performed in which the pH microelectrode was replaced by a similar micropipette filled with 2 M NaCl (see Results). pH electrodes were fitted with Ag-AgCl junctions that fed a high input impedance (>\(10^{13}\) \(\Omega\)) head stage. Surface pH electrode records were elicited at 15 s intervals and filtered at 2 kHz.
Antidromic Stimulation of the CA1 Neuronal Population.

Constant current pulses of 300 µs duration were delivered to the alveus by a pair of 50 µm diameter, Teflon-insulated platinum-iridium wires in saline containing 10 µM DNQX, 50 µM APV and 100 µM picrotoxin. These agents insured that activation did not occur via synaptic transmission. This was confirmed by monitoring of the short-latency, extracellular, antidromic population spike.

Simulated Antidromic Invasion.

To determine whether a train of action potentials could evoke a surface pH shift, we devised a paradigm to simulate the voltage response of a neuron to repetitive antidromic activation. First, we recorded the whole cell current clamp response of a CA1 pyramidal neuron to antidromic stimulation of the alveus at 100 Hz for 2 seconds (see above). The waveform then served as the voltage command to evoke surface pH shifts on other cells.

Recording of Population pH$_e$ Responses.

The concentric pH microelectrode and a separate reference micropipette were mounted on a dual micromanipulator, with a tip separation of 5-10 µm, as described by Fedirko et al. (2006). The array was then advanced into the CA1 cell body layer until the antidromic population spike was maximal, which typically occurred at a depth of 100 –
150 µm. Capacitance neutralization was used to match the time constant of the reference and pH electrodes, as judged by the response to a 1 ms, 1 mV calibration pulse in the common ground circuit. Slow DC potentials recorded on the reference barrel were continuously subtracted from the pH recording to yield the H⁺ signal. Trains of stimuli were delivered every 2 min. to obtain raw traces.

Data Analysis and Presentation

Statistics were presented as means with standard error. Values of n refer to the number of neurons or slices studied, as indicated. Dual comparisons between mean values were made with a two-tailed, Student's paired t test. Multiple comparisons were made using paired ANOVA with a Student-Newman-Keuls post hoc test. Three to five raw traces of all responses were averaged before data analysis. In all records, alkaline shifts were presented as upward deflections.
Results

Surface Measurements from CA1 Pyramidal Neurons

In all experiments the holding potential was -60 mV. Initial studies were performed on ten CA1 pyramidal neurons. With the pH electrode placed against the cell soma, its voltage response ($V_{pH}$) to a 2 s. depolarizing step to 0 mV was dominated by a rapidly rising negativity (Figure 1, top row, left), with a peak amplitude of -1.08 ± 0.17 mV (equivalent to an alkalosis of 0.019 ± 0.003 unit pH). Termination of the voltage clamp step was followed by a roughly exponential decline of $V_{pH}$ toward baseline. With inclusion of APV and DNQX in the saline (to block NMDA and AMPA receptors, respectively), plus addition of Cs⁺ and QX-314 to the patch solution (to respectively inhibit K⁺ and Na⁺ channels), similar surface responses could be elicited (n=4 cells, data not shown).

Brain slice experiments that employ ion sensitive electrodes typically make use of a second reference barrel, to enable subtraction of common DC voltages. Such potentials can arise from real physiological responses across the extracellular space (Somjen, 1973), or from simple voltage drops across the bath and ground pathway during current passage. In the present study, which utilized concentric ion-selective microelectrodes on the cell surface without an accompanying reference pipette, it was therefore essential to first determine whether such slow potentials significantly contaminated $V_{pH}$.

At the start and end of the voltage clamp pulse, small, brief, positive and negative deflections of $V_{pH}$ were consistently noted (Figure 1, top row, arrows), suggestive of a
minor DC component. To examine this further, we withdrew the pH microelectrode from the somata in steps. The large, negative component of $V_{\text{pH}}$ was reduced by about half at 5 µm from the cell membrane, and disappeared altogether by 10 µm. However, a small, positive, rectangular DC deflection appeared, and remained evident, even as the electrode was withdrawn from the slice into the overlying saline, millimeters away from the voltage-clamped cell (Figure 1, top row, right). This indicated that the DC deflections represented a voltage drop across the current path from bath to ground. To further confirm this, we recorded surface potentials by replacing the pH electrode with a 2 M NaCl-filled reference micropipette ($V_{\text{Ref}}$). Here, the voltage step to 0 mV produced similar, small ($90 \pm 50 \mu\text{V}$), positive, rectangular, DC shifts ($n = 6$ cells) at the cell surface, which remained unchanged during withdrawal of the micropipette through the slice, and into the bath (Figure 1, middle row). We were therefore confident that the on-off transients on the raw $V_{\text{pH}}$ records were due to the same phenomenon, and unrelated to changes in pH.

Since these DC artifacts were less than 10 percent of the $V_{\text{pH}}$ responses, rectangular in waveform, and consistent throughout a given experiment, we removed the transients from the $V_{\text{pH}}$ traces. This was done by simple upward displacement of the records during the voltage clamp pulse. With the exception of Figure 1, the displayed surface records were all corrected in this manner. A corrected recording is shown in Figure 2A along with a photograph illustrating the placement of the pH and patch electrodes on a soma.
Somatic depolarization also evoked surface $V_{pH}$ responses from the proximal dendrite, as shown in Figure 2B. This response, however, was smaller (-0.30 to -0.50 mV) and less reliably obtained, as it was difficult to visually match a given soma to the correct proximal dendrite. We therefore concentrated our efforts on the somatic responses.

The Effect of Altering Extracellular Buffering Capacity

To confirm that the response of the surface pH electrodes represented a real change in pH, we manipulated the extracellular buffering capacity. Buffering was increased by the addition of HEPES to the standard bicarbonate-buffered ACSF (which contained benzolamide). In control ACSF, a voltage step to 0 mV caused a mean peak surface shift of -0.74 ± 0.16 mV that was reduced by 43 ± 7.3 percent after the addition of 1 mM HEPES (n=5 cells; p < 0.01), as shown in Figure 3A. With addition of 5 mM HEPES, the control responses (-0.65 ± 0.08 mV) were reduced by 90.9 ± 5.7 percent (n=5 cells; p < 0.001; Figure 3B). Finally, with addition of 20 mM HEPES, the control response (-0.64 ± 0.12 mV) was reduced by 98.8 ± 1.2 percent (n=7 cells; p < 0.01; Figure 3C). The effects of the HEPES solutions are summarized in Figure 3D.

Effective buffering capacity was decreased by the addition of benzolamide to the ACSF (Tong et al., 2006). These were the only experiments in which the control solution did not contain the carbonic anhydrase inhibitor. In the control ACSF, a step depolarization to 0 mV caused a peak surface shift of -0.53 ± 0.11 mV (n=5 cells), which corresponds to a pH shift of roughly 0.01. The addition of benzolamide reversibly
increased the peak response by 356 ± 100 percent (range 137 – 703 percent; p<0.05; Figure 3E). The effect of benzolamide is summarized in Figure 3F.

The changes noted with manipulation of buffering power were not due to run-down or run-up of the responses, as simple delays of 5 min., corresponding to wash in time, had no significant effect on the amplitude of the surface alkalinizations (n=6 cells; p=0.56; Figure 3F). Thus, these data collectively indicated that the pH electrode responses were indeed surface alkaline transients (SATs).

The Effect of Membrane Potential on the SAT

The peak magnitude of the SAT was studied over a range of voltage clamp steps from -120 up to +40 mV, in 20 mV increments. Representative traces are displayed in Figure 4A. Hyperpolarizing steps produced no observed surface pH change (Figure 4AB). The SAT was greatest at 0 mV, and declined for steps to +20 and +40 mV (Figure 4B, n = 10 cells; repeated measures ANOVA with Student-Newman-Keuls post hoc test). These data are consistent with the SAT being caused by a transporter. Were it mediated instead by a conductive pathway, one would predict the alkalosis to reverse into an acidosis at voltages more positive to the equilibrium potential for H+ (E_{H+}) (Roos and Boron, 1980; Chesler, 1990). Assuming a pH_e of 7.2 in the mouse hippocampal slice (Tong et al., 2000), and a pH_i of 7.3, E_{H+} would have been approximately +6 mV. Since the SAT was maximal at 0 mV, and remained alkaline at +40 mV, the response could not have arisen due to a flux of H+, or one of its acid-base equivalents, through a channel.
Dependence of the SAT on Extracellular Ca\(^{2+}\)

The PMCA is activated following entry of Ca\(^{2+}\). With elevation of cytosolic Ca\(^{2+}\), calmodulin binds to the PMCA, relieving the suppressive effect of an auto-inhibitory domain located on the C terminus of the transporter (James et al., 1988; Falchetto et al., 1991; 1992). If the SAT of hippocampal neurons was caused by the PMCA, then one would expect a diminished response if the influx of Ca\(^{2+}\) were inhibited. In experiments on six neurons, the addition of 300 µM Cd\(^{2+}\) to the extracellular fluid reduced the control SAT (0.014 ± 0.002 unit pH) by 71 ± 14.0 percent (p<0.01; data not shown). Since Cd\(^{2+}\) can independently inhibit the PMCA (Akerman et al., 1985; Verbost et al., 1988), we also tested nifedipine, a more specific blocker of high-threshold L-type Ca\(^{2+}\) channels. Nifedipine (25 µM) reduced the control SAT (0.019 ± 0.004 unit pH) by 53 ± 9.0 percent (n = 6 cells; p < 0.01; Figure 5A). In saline with zero added Ca\(^{2+}\) (plus 1 mM EGTA), the control SAT (0.032 ± 0.010 unit pH) was completely abolished (n=4 cells; p<0.05; Figure 5B). Thus, generation of the SAT required the entry of Ca\(^{2+}\) ions.

To suppress a rise in intracellular Ca\(^{2+}\), we included the Ca\(^{2+}\) chelator BAPTA (1 mM) in the patch pipette, and recorded surface responses 1 min. and 5 min. after breakthrough into whole cell mode. At 1 min., the SAT measured 0.009 ± 0.002 unit pH, and was reduced by 79 ± 7.2 percent after 5 min. (n = 5 cells; p < 0.05). An experiment in which BAPTA abolished the SAT is shown in Figure 5C. These data indicated that the generation of the SAT was dependent on a rise in cytosolic Ca\(^{2+}\). The effects on the SAT
due to inhibition of Ca\textsuperscript{2+} entry, or suppression of the cytosolic Ca\textsuperscript{2+} rise, are summarized in Figure 5D.

The Effect of PMCA inhibitors on the SAT

Carboxyeosin (CE) is a relatively potent blocker of the PMCA that acts on the cytoplasmic side of the transporter, with a reported K\textsubscript{i} of 20 nM (Gatto and Milanick, 1993). Due to the charged nature and poor membrane permeability of this compound, we included it in the patch pipette to determine the contribution of the PMCA to the SAT. While eosin derivatives are known to also block Ca\textsuperscript{2+}/H\textsuperscript{+} exchange across the ER and mitochondria (O’Neal et al., 1979; Kosterin et al., 1996; Watson et al., 2003), inhibition of these mechanisms would not decrease a surface alkalosis. With 0.5 µM CE in the filling solution, the mean SAT at 1 min. measured 0.007 ± 0.001 unit pH, and was reduced by 86 ± 4.8 percent after 5 min (n = 8 cells; p <0.01; Figure 6A, left). With 5 µM CE in the pipette, the SAT measured 0.010 ± 0.003 unit pH at 1 min, and was completely abolished after 5 min. (n=10 cells; p < 0.01; Figure 6A, right). After 5 min. of dialyzing the cells with 5 µM CE, neither the input resistance, nor the holding current, were significantly changed from their values at 1 min. Thus, the suppression of the SAT was not due to a non-specific loss of cell viability.
Although CE in the patch pipette was appropriate for blocking the SAT, we sought a means of inhibition via an extracellular approach, as this would be necessary for a comparison against the population response (see below). The only available pharmacologic tools that block the PMCA at an extracellular locus are the caloxin peptides. We tested caloxin 2A1, with a reported $K_i$ of 0.4 mM (Chaudhary et al., 2001). In experiments on six pyramidal neurons, superfusion of 2 mM caloxin reduced the control SAT (0.017 ± 0.003 unit pH) by 42 ± 10.2 percent ($p<0.05$; Figure 6B). The addition of caloxin had no effect on the input resistance or holding current in these experiments, thus it was unlikely that a loss of cell viability was responsible for the reduction in the SAT.

Effects of intracellular CE, and extracellular caloxin on the SAT are summarized in Figure 6C.

Simulated Antidromic Invasion Evokes an SAT

To determine whether the transient depolarizations associated with a repetitive spike train could also elicit an SAT, a series of experiments was carried out to simulate antidromic firing. Here, we used the whole-cell current clamp response to a 100 Hz, 2 s. antidromic stimulus train (previously obtained from a different cell; Figure 7A, left) as the voltage clamp command. This protocol had advantages over the injection of constant current since it avoided irregularity in responses (e.g., due to accommodation), and thus insured a standardized sequence of "spikes" for all cells studied. It also allowed for a meaningful comparison with a PAT elicited by real antidromic stimulation (see below). The simulated antidromic train elicited an SAT with a peak amplitude of 0.017 ± 0.004 unit
pH (n=12 cells; Figure 7A, right), similar to the surface responses evoked by a 2 s. step depolarization to 0 mV.

Simulated Antidromic SAT vs. Antidromic Population Response

In order to compare the previously obtained SATs evoked by simulated antidromic invasion of one neuron, against the pH_e response from the CA1 neuronal population, we stimulated the alveus at 100 Hz for 2 s. and recorded the resulting population alkaline transient (PAT) in stratum pyramidale (n=8 slices). Thus, in these PAT experiments, the stimulus duration and frequency, as well as the placement of the pH electrode, were identical to those in the SAT experiments. The only major difference between the two paradigms was the method by which neurons were activated (simulated vs. real antidromic invasion). Since accurately measuring PATs requires that pH and reference electrodes be used simultaneously, these experiments were conducted in different slices than those in which SATs were recorded (above).

The SAT previously elicited by a 100 Hz, 2 s. train was evident at 200 ms after the start of the stimulus, at which time it measured 0.0012 ± 0.0005 unit pH. This value was 12 percent of the comparable magnitude of the PAT at 200 ms, which measured 0.010 ± 0.002 unit pH (Figure 7B). These relative measures were similar at the peak of the two responses. The mean peak amplitude of the SAT had measured 0.017 unit pH (see above), which was 11 percent of the mean peak amplitude of the PAT (0.155 ± 0.030 unit pH; Figure 7C). Like the SAT, the PAT began to decline almost immediately after termination of the stimulus
train, however its decay was slower. The relaxation of the SAT had a half-time of $1318 \pm 179$ ms, while the PAT decayed with a half-time of $2715 \pm 267$ ms (Figure 7D).

Effect of PMCA Inhibitors on the Simulated Antidromic SAT and Antidromic PAT

The SAT evoked by simulated antidromic firing was suppressed by inhibitors of the PMCA. With 5 µM CE in the patch pipette, the “antidromic” SAT was virtually abolished. The control response averaged $0.014 \pm 0.005$ unit pH at 1 min, and was reduced by $99 \pm 1$ percent after 5 min of dialysis with CE ($n = 5$ cells; $p < 0.05$; Figure 8A). A control SAT of $0.010 \pm 0.002$ unit pH was reduced by $43 \pm 12$ percent after addition of 2 mM caloxin ($n=5$ cells; $p<0.05$; Figure 8B).

To evoke a population alkaline response, CA1 axons were activated antidromically (with a 20 pulse, 50 Hz stimulus train), and pH$_e$ was recorded in stratum pyramidale, as above. The control PAT ($0.068 \pm 0.007$ unit pH) was inhibited by $42 \pm 4.7$ percent after superfusion of 2 mM caloxin ($n=5$ slices; $p<0.001$; Figure 8C). Thus, the effects of caloxin on the PAT and SAT were roughly identical. Caloxin had no effect on the extracellular population spike, and in current clamp recordings of antidromic invasion from two cells, neither the spike height, nor the number of spikes was reduced (data not shown).

Therefore the suppression of the PAT by caloxin could not be attributed to a reduction in excitability.
Discussion

A major result of this investigation was the demonstration of an alkalosis at the surface of singly activated CA1 pyramidal neurons, elicited by both depolarizing voltage clamp steps, and simulated, repetitive firing. Our data indicated that the PMCA was the sole generator of this SAT, and was most likely the underlying mechanism of the extracellular alkaline shift evoked by synchronous activation of this neuronal population. It has been previously shown that the PAT requires entry of extracellular Ca\(^{2+}\) (Grichtchenko and Chesler, 1996; Paalasmaa and Kaila, 1996). Similarly, the SAT required the presence of external Ca\(^{2+}\), as well as its entry and elevation in the cytosol, and the reduction of the SAT at positive holding potentials was consistent with a lower driving force for Ca\(^{2+}\) entry. In addition, the fractional magnitude of the response from a singly activated neuron relative to that of the population response was striking, and provided additional evidence that PMCA-mediated surface responses were the likely basis for the PAT.

Inhibition studies further supported the PMCA hypothesis. These experiments were necessarily limited to pharmacological approaches. Since there are four principal isoforms of the PMCA, with numerous splice variants (Strehler and Zacharias, 2001) knock down or knock out strategies could not be readily implemented. Indeed, isoforms 2, 3 and 4 are all expressed on hippocampal pyramidal neurons (Kip et al., 2006). Available pharmacological tools to block the PMCA have certain drawbacks that warrant discussion, however.
In addition to blocking the PMCA, CE also inhibits organellar Ca\textsuperscript{2+}-ATPases (O’Neal et al., 1979; Kosterin et al., 1996; Watson et al., 2003). Activity of these transporters would normally acidify the cytosol. The PMCA, however, is the only known mechanism that would be expected to generate a surface alkalosis that was reduced by internal CE. Thus, the activity and inhibition of the PMCA was the most obvious and parsimonious explanation for the surface pH data.

Similarly, the reduction of the SAT by the bath application of the peptide caloxin cannot be explained by an intracellular action. While the $K_I$ of caloxin is relatively high (0.4 mM), it is currently the most effective extracellular PMCA blocker. Importantly, caloxin reduced the SAT and PAT to a similar degree (42 percent). This partial block was expected, given that application of 2 mM caloxin did not completely inhibit the PMCA response in the peptide’s original description (Chaudhary et al., 2001). To avoid potential non-specific effects, we did not add higher concentrations of this agent. Since caloxin did not affect antidromic spike invasion, field potentials, or input resistance, its effect on the PAT is best explained by inhibition of the PMCA.

In giant snail neurons, Schwiening and colleagues (1993) reported a rise of surface pH attributable to the PMCA, and first suggested that a similar response might be the basis for the PATs that had been reported in certain regions of mammalian brain. This attractive hypothesis was supported by reports of the Ca\textsuperscript{2+}-dependence of the PAT.
(Grichtchenko and Chesler, 1996; Paalasmaa and Kaila, 1996). However, unlike the PAT in hippocampal slices, which was evident within tens of milliseconds (Tong et al., 2006), the SAT of snail neurons was barely detectable after 2 seconds of depolarization (e.g. Figure 4 of Schwiening et al., 1993). The onset kinetics of SATs were also obscured in later studies of skate (Molina et al., 2004) and catfish (Kreitzer et al., 2007) horizontal cells. Thus, to address the relationship between a neuronal SAT and the PAT of mammalian brain slices, improved temporal resolution was required.

In the present study, use of concentric pH microelectrodes enabled detection of an SAT on single hippocampal pyramidal neurons \textit{in situ}, and allowed for a comparison with the antidromic PAT as early as 200 ms after onset of stimulation. Using a 2 s, 100 Hz, simulated, antidromic stimulus, the average surface response of a single pyramidal cell amounted to one-ninth the response elicited when the neuronal population was similarly activated. Under normal buffering conditions (i.e., in the absence of benzolamide) the SAT was also a sizable fraction of a PAT: Although the SAT averaged just 0.01 unit pH under these conditions, the comparable antidromic PAT in the absence of benzolamide is typically in the range of 0.05 – 0.10 unit pH (e.g. Chen and Chesler, 1992b; Grichtchenko and Chesler, 1996; Shah et al., 2005).

The principal issue raised by these results is whether the PMCA responses from single neurons can combine to produce a larger PAT. It can be intuitively argued that the greater the number of cells activated, the greater will be the amount of net acid removed
from the local extracellular volume. Since H\(^+\) influx via the PMCA generates extracellular base instantly from water, it may equivalently be called a base source. With activation of a single neuron, the base source would become highly diluted and diminished within the gross extracellular space, as was evident in the experiment of Figure 1, where the pH electrode was gradually withdrawn from the cell surface. By contrast, with activation of the population, more total base would be added, while diffusion away from the surface of each cell would be curtailed (due to a less steep concentration gradient). Accordingly, the essential differences between an SAT and a PAT would arise due to the contrasting effects of diffusion when activating single vs. multiple point sources.

This concept can be conveyed using a highly simplified model of concentration changes at one locus, in the context of single or multiple point sources. Here we consider one vs. three sources located one micron from a point of measurement (Figure 9A, left two panels respectively) then add an additional 20 or more sources located 15 microns distant (Figure 9A, right panel). For an arbitrary diffusible ion generated by a source \(Q\) of fixed amplitude (in mol-sec\(^{-1}\)), the concentration \((C)\) at time \(t\) and distance \(r\) is given by

\[
C(r,t) = \frac{Q}{4\pi Dr} \text{erfc}\left(\frac{r}{2\sqrt{Dt}}\right),
\]

where \(Q\) is the source in mol-sec\(^{-1}\), \(D\) is the diffusion coefficient in cm\(^2\)-sec\(^{-1}\), and \text{erfc} is the complementary error function (Nicholson and Phillips, 1981). The concentration rise at 1 micron from a source turned on for 204 ms is illustrated by the gray lower trace in Figure
If three such sources are placed 1 micron from the point of measurement, the responses sum, producing a faster rate of rise and a three-fold larger peak. With an additional 20 sources placed 15 microns from the point of measurement, the response again grows in magnitude, but only modestly. Addition of a total of 100 sources at 15 microns causes little further increase in the magnitude.

The small effect of the additional sources can be attributed to their distance from the point of measurement. The latency of diffusion limits their contribution at early times, and their early termination occurs before their effect can fully manifest. Another property conferred by the activation of multiple point sources is the slowing of the decay, as shown in the normalized recoveries in the inset of Figure 9B. This is attributable to a less steep concentration gradient than would occur with activation of a single point source.

The generation and spread of an alkaline shift is a more complex phenomenon. It is recorded as a pH (rather than concentration) change, which is generated by base sources distributed over the complex surface area of cells. In addition, it manifests by a process of reaction-diffusion involving mobile buffer, and may be regulated by glial cells (Chesler 1990; Ransom 1992; Rose and Deitmer, 1994). It would nonetheless be subject to the same principles of diffusion that pertain in a simpler system. Accordingly, the response would be expected to grow in magnitude as more local sources were activated, whereas more distant sources would contribute less to the peak, but would slow the recovery phase.
These concepts are consistent with both the larger amplitude of the PAT, and its slower recovery compared with the SAT (Figure 7).

The rapid PAT observed in hippocampal slices also occurs in the hippocampus in vivo (Somjen, 1984; Xiong and Stringer, 2000), and a similar alkalosis that was amplified by a carbonic anhydrase inhibitor (acetazolamide) was first reported in rat cerebellum (Kraig et al, 1983). However, it should be pointed out that activity-dependent alkaline responses do not all arise via this proton sink mechanism. In retina, stimulation by light caused an alkaline shift due to a fall in ongoing production of metabolic acid (Yamamoto et al., 1992). In the caudate nucleus, an activity dependent alkalosis diminished by acetazolamide was correlated with hyperemia, consistent with increased washout of CO2 (Venton et al., 2003).

In addition, a rise in pHe is not the universal response to neural activity throughout the central nervous system. In rat parietal cortex, synchronous activity caused a slowly developing acidosis (Chesler and Kraig, 1989) and the response in adult spinal cord was dominated by a rapidly rising acid shift (Sykova and Svoboda, 1990). These differences are likely due to any number of region-specific factors, such as heterogeneity in the expression of the PMCA, voltage-gated Ca\textsuperscript{2+} channels, and various acid-base transporters on neurons and glia (Deitmer and Rose, 1996; Chesler, 2003).

The functional significance of the hippocampal PAT was highlighted in a recent study (Makani and Chesler, 2007) in which augmentation of extracellular buffering was found to curtail post-synaptic responses mediated by NMDARs, which are inhibited by
external protons in the physiological pH range (Traynelis and Cull-Candy, 1990; Tang et al., 1990; Vyklicky et al., 1990). Similarly, an earlier report noted that epileptiform discharges of hippocampal slices were suppressed by addition of HEPES to the bathing fluid (Taira et al. 1993). These results suggest that following synchronous excitatory synaptic activity, alkaline shifts in the micro-domain of the post-synaptic membrane can rapidly attain a magnitude sufficient to relieve the proton block of NMDA receptors. In view of the NMDAR titration curve (Traynelis and Cull-Candy, 1990; Tang et al., 1990; Vyklicky et al., 1990), these studies suggested that the magnitude of the alkaline shift at the sub-synaptic membrane was considerably larger than the pH shifts recorded from the larger volume of the extracellular space by microelectrodes.

This inferred effect of the PMCA on sub-synaptic extracellular pH is consistent with reports of PMCA localization. While this transporter is ubiquitous, particularly dense staining for the PMCA was associated with the post-synaptic densities of dendritic spines in cerebellar Purkinje neurons (Hillman et al., 1996). In addition, the PMCA2b splice variant was found to co-localize with synapse associated protein 90/PSD95 at individual dendritic spines of CA1 pyramidal neurons (DeMarco and Strehler, 2001). These postsynaptic proteins have been reported to also interact with NMDARs (Niethammer et al., 1996), suggesting that PMCA and NMDARs are in close proximity. Accordingly, the PMCA may be able to generate large alkaline shifts in the immediate extracellular microdomain of NMDA receptors.
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**Figure Legends**

**Figure 1.** Distance profile of traces from pH-sensitive vs. saline-filled microelectrodes.

Top panel shows surface pH electrode responses to a depolarizing voltage step from -60 to 0 mV, at various distances from the cell body of a pyramidal neuron. Arrows indicate transient voltage artifacts. Middle panel (gray) shows the respective profile obtained when a 2 M NaCl-filled reference pipette replaced the pH microelectrode. Note that the small, downward, rectangular deflection was common to both electrodes and present at all distances. Alkaline responses are indicated by upward deflections in this and all subsequent figures. All solutions contained benzolamide unless noted otherwise.

**Figure 2.** Surface pH electrode responses observed at soma and dendrite. The pH microelectrode was placed either opposite the patch pipette on the soma (A) or on the proximal dendrite (B). Recordings and photographs in A and B are from different slices. sr: s. radiatum; sp: s. pyramidale; so: s. oriens; Vm: patch pipette. The calibration bars represent 10 µm.

**Figure 3.** pH electrode response is a surface alkaline transient (SAT). A. Addition of 1 mM HEPES to the bicarbonate-buffered media significantly reduced the SAT. B. The addition of 5 mM HEPES suppressed most of the SAT. C. Addition of 20 mM HEPES completely eliminated the SAT. D. Summary of HEPES data. All statistical comparisons were paired to respective controls. E. Comparison of SAT before (Ctl) and after superfusion of 10 µM
benzolamide (Bz). F. Mean effect of a 5 min. delay, or application of Bz, compared against respective controls.

Figure 4. Surface alkaline transient varied with voltage. A. Selected SATs from one cell at various step potentials. B. Mean SAT peak amplitudes in response to step potentials ranging from -120 to +40 mV. Mean response at 0 mV was significantly greater than that at all other potentials (repeated measures ANOVA with Student-Newman-Keuls post hoc test). Note the surface alkalinization never reversed into an acid shift.

Figure 5. Surface alkaline transient requires entry of Ca²⁺. A. Comparison of superimposed SATs before and after the addition of nifedipine (25 µM). B. Comparison of SATs in control solution (Ctl) and after Ca²⁺ was removed from the ACSF. C. BAPTA (1 mM) included in the patch pipette greatly reduced the SATs. The SAT responses were compared at 1-min and 5-min after breakthrough into whole-cell mode. D. Mean effects of manipulating the entry of Ca²⁺ or its cytosolic rise. All statistical comparisons were paired against respective controls.

Figure 6. Surface alkaline transient is blocked by PMCA inhibitors. A. Carboxyeosin (CE) was included in the patch pipette solution at a concentration of either 0.5 µM (left) or 5 µM (right). SATs were measured at 1-min and 5-min after breakthrough into whole-cell mode. B. Comparison of SATs in control solution (Ctl) and after the addition of caloxin (2 mM).
C. Mean effects of PMCA inhibitors on the SAT. Comparisons were paired against respective controls.

Figure 7. Comparison of responses evoked by simulated vs. real antidromic activation. A. The whole-cell current-clamp response (left) to 100 Hz (2 sec) stimulation of CA1 axons was used as the voltage command to generate SATs in later experiments (right). B. Overlay of initial rise of the SAT and PAT. C. Overlay of an SAT generated by antidromic simulation with a PAT generated by antidromic stimulation in a different slice. Both traces were elicited by a 100 Hz, 2 s. train. D. Example of the normalized decays of an SAT versus a PAT.

Figure 8. Effect of PMCA blockers on responses evoked by simulated vs. real antidromic activation. A. Carboxyeosin (5 µM) was included in the patch pipette solution, and SATs were generated by antidromic simulation at 1-min and 5-min after breakthrough into whole-cell mode. B. Comparison of a control SAT evoked by antidromic simulation and after the addition of caloxin (Clx) (2 mM) to the ACSF. C. PATs were evoked by a 20 pulse, 50 Hz, real antidromic stimulus train. Overlay of control PAT (Ctl) and after the superfusion of caloxin (Clx) is shown.

Figure 9. Concentration changes for a monovalent ion originating from single vs. multiple point sources. A. Left - single point source with point of measurement (*) at 1 µm; middle - three point sources at 1 µm; right – addition of another 20 point sources at 15 µm. B.
Concentration changes for ion resulting from one point source at 1 μm (lower gray), three point sources at 1 μm (upper gray), additional 20 sources at 15 m (lower black), or an additional 100 sources at 15 μm. Numerals under traces denote number of sources. Inset shows normalized decay of two traces indicated by corresponding numerals. Curves were calculated using $D = 10^{-6} \text{cm}^2\text{sec}^{-1}$, and a step point current source of 10 μA turned on for 204 ms.
A B C D

% Control

0 50 100

Nif 2 s

Ctl Cd 2+

BAPTA 1 min 5 min

pH 0 Ca^{2+}

.01 ΔpH

.02 ΔpH

.01 ΔpH

.01 ΔpH

0 Ca^{2+}

Ctl Ca^{2+} Nif 0 Ca^{2+} BAPTA

* **
**Carboxyehosin in pipette**

**A**

1 min

5 min

\[ \text{pH} \]

\(0.1\)

\(2\) s

\(\Delta \text{pH}\)

1 min

5 min

B

\(\text{Ct}\)

\(\Delta \text{pH}\)

\(5\) min

\(2\) s

C

\(\text{Ctl}\)

\(\text{Caloxin}\)

% Control

**C**

5-min

CE

** **

\(0.5\) \(\mu\)M

\(5\) \(\mu\)M

% Control
A SAT Carboxyeosin 5 min
.01 ΔpH
.02 ΔpH
1 min 5 min
2 s

B SAT Ctl Clx
.005 ΔpH

C PAT Ctl Clx
.02 ΔpH
2 s
A

3 sources @ 1 μm

1 source @ 1 μm

+20 sources @ 15 μm

B

$r = 1 \, \mu m$

$r = 15 \, \mu m$

μM

0 100 200 300 400 ms