

# Temporal Resolution of Activity-Dependent pH Shifts in Rat Hippocampal Slices

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## SUMMARY AND CONCLUSIONS

1. The rise time of activity-dependent extracellular pH shifts was measured in the CA1 stratum radiatum of rat hippocampal slices by recording pH-sensitive fluorescence of a fluorescein-conjugated dextran. Optical data were compared with simultaneous pH microelectrode recordings.

2. The pH shifts generated by CO<sub>2</sub> or by stimulation of the Schaffer collaterals were paralleled by shifts in fluorescence emissions at 535 nm when the probe was excited with 490-nm light ( $\Delta F_{490}$ ). Emissions at 535 nm induced by 440-nm light were unchanged in these paradigms.

3. A train of three stimuli at 100 Hz was repeated at 30-s intervals and the stimulus-triggered  $\Delta F_{490}$  was averaged. The mean rise time of the  $\Delta F_{490}$  was  $69 \pm 24$  (SE) ms (range 20–200 ms,  $n = 6$ ). The mean increase in emission was  $0.75 \pm 0.22\%$  of baseline, associated with a pH microelectrode response of  $+0.06 \pm 0.02$  unit pH.

4. These data demonstrate that synaptically evoked alkaline transients develop within tens of milliseconds. The occurrence of the alkalization in the same time frame as excitatory postsynaptic currents indicates that these pH shifts arise with sufficient speed to modulate synaptic transmission.

## INTRODUCTION

Alkalinization of the extracellular space is a frequent result of neuronal activity (Chesler 1990; Chesler and Kaila 1992; Kraig et al. 1983). These shifts are especially robust in hippocampus, attaining magnitudes up to 0.2 unit pH during repetitive stimulation of the Schaffer collaterals (Carlini and Ransom 1986) and a fraction of this amplitude in response to single orthodromic stimuli (Gottfried and Chesler 1994). In a previous report, lowering the extracellular buffering capacity with benzolamide, a carbonic anhydrase inhibitor, caused a gradual enhancement of orthodromic field potentials (Taira et al. 1993). It was subsequently shown that the decreased buffering amplified the alkaline shifts induced by single shocks to the Schaffer collaterals and prolonged the time course of concomitant *N*-methyl-D-aspartate (NMDA)-receptor-mediated excitatory postsynaptic currents (EPSCs) (Gottfried and Chesler 1994). These results suggested that endogenous alkaline transients can modulate synaptic transmission due to the pH sensitivity of postsynaptic NMDA receptors (Tang et al. 1990; Traynelis and Cull-Candy 1990; Vyklicky et al. 1990).

To influence a single EPSC, an alkaline shift must occur within a few tens of milliseconds. Using phenol red as a probe of extracellular pH (pH<sub>o</sub>), Krishtal and colleagues (1987) noted an alkaline shift that peaked ~150 ms after stimulation of the Schaffer collaterals. Although these data

would suggest that the alkaline shift is too slow to modulate synaptic responses, these previous experiments were conducted at room temperature. We therefore sought to measure the onset of the alkaline transients at a more elevated temperature, under conditions similar to those in which a neuromodulatory role was inferred (Gottfried and Chesler 1994). To resolve the onset of the pH changes, we used a dextran-conjugated fluorescein probe injected into the extracellular space of rat hippocampal slices. Our data demonstrate a far more rapid onset of the alkaline shift than has previously been noted. These results indicate that the extracellular alkalization can develop with sufficient speed to modulate postsynaptic currents.

## METHODS

Thin hippocampal slices (150–200  $\mu$ m), prepared from methoxyflurane-anesthetized Long-Evans rats (postnatal day 12–17), were placed in a submersion-style chamber mounted on an inverted microscope equipped for epifluorescence ( $\times 40$  dry objective, 0.75 numerical aperture). Preparations were superfused with Ringer solution (32°C) containing (in mM) 124 NaCl, 3 KCl, 26 NaHCO<sub>3</sub>, 10 glucose, 1 Na<sub>2</sub>HPO<sub>4</sub>, and 3 CaCl<sub>2</sub>, pH 7.4, gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub>. To maximize alkaline shifts, Mg<sup>2+</sup> was omitted and 2  $\mu$ M benzolamide (Lederle Laboratories) was added (Gottfried and Chesler 1994). Picrotoxin (100  $\mu$ M) was included (Sigma Chemical, St. Louis, Mo.) to prevent  $\gamma$ -aminobutyric acid-A receptor-mediated pH shifts (Chen and Chesler 1992; Kaila et al. 1992). The Schaffer collaterals were activated with a pair of 50- $\mu$ m insulated platinum-iridium wires. Fabrication and use of double-barreled pH microelectrodes have been described (Chesler and Chan 1988).

A 2,000,000-MW dextran conjugated to fluorescein was used to resolve small, fast optical signals resulting from rapid pH<sub>o</sub> shifts (Molecular Probes, Eugene, OR, cat. No. D-7137). The probe was dissolved in 10 mM Na<sub>2</sub>HPO<sub>4</sub> (2.5  $\mu$ M at pH 7.3) and microinjected into the CA1 stratum radiatum (within 10  $\mu$ m of a pH microelectrode) until baseline fluorescence stabilized. Thereafter, emissions decayed by ~1% per minute, indicating poor diffusion and limited photobleaching of the probe (Nicholson and Tao 1993). Thus, over the maximal measurement interval of ~25 min, the baseline fluorescence decayed by roughly one fourth. Throughout the experiments the microscope objective was focused on the tip of the pH microelectrode. Thus the light of excitation and the source of emitted fluorescence were confined mainly to the focal plane that contained the microelectrode tip. The depth of field, defined as the distance between the principal minima of the Z-axis point spread function, would be roughly 2  $\mu$ m for a  $\times 40$  dry objective with a numerical aperture of 0.75 (Tao and Nicholson 1995). The tag was excited using a 75-W xenon arc lamp at either 490  $\pm$  10 nm, the wavelength at which emission is highly pH sensitive, or 440  $\pm$  10 nm, the wavelength at which emission is pH insensitive (Tsien 1989). Emissions at 535 nm were monitored

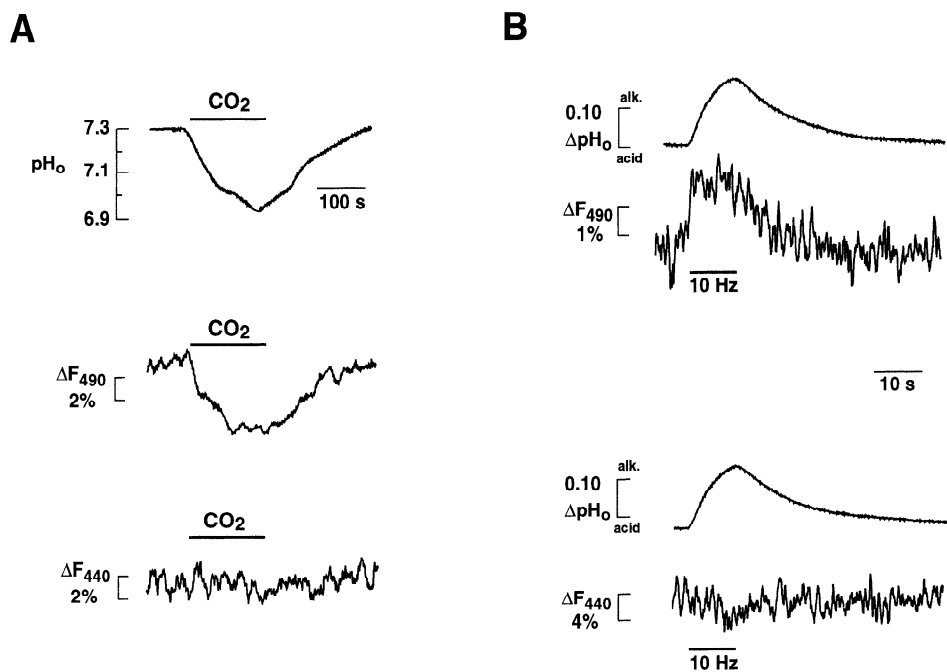


FIG. 1. Detection of slow pH shifts with dextran-conjugated fluorescein. *A*: exposure to 100% CO<sub>2</sub> generated an extracellular acid shift. *Top trace*: pH microelectrode response. Fluorescence emission evoked at 490 nm ( $\Delta F_{490}$ ) decreased with a comparable time course (*middle trace*). Fluorescence emission evoked at 440 nm ( $\Delta F_{440}$ ) was unchanged (*bottom trace*). *B*: repetitive stimulation of the Schaffer collaterals. The pH microelectrode detected an extracellular alkaline shift (averaged from 5 trains).  $\Delta F_{490}$  displayed a correlated increase (*top pair of traces*). A subsequent 5 trains evoked a similar pH microelectrode response, with no shift in the  $\Delta F_{440}$  (*bottom pair of traces*).

with a photomultiplier fitted with a movable shutter that limited light collection to a 100- to 500- $\mu\text{m}^2$  window above the stratum radiatum (Photon Technology International). Endogenous background fluorescence of the tissue was roughly 5% of baseline and was not subtracted. Excitation and emission filters were supplied by Omega Optical (Brattleboro, VT).

With 250- to 500-Hz sampling, the individual evoked responses were obscured by high-frequency noise, requiring filtering and stimulus-triggered averaging of  $\geq 50$  traces. This limited the temporal resolution to  $\sim 5$  ms. Thus differences in conduction time and synaptic delay, which varied by  $\sim 0.5$  ms, had no significant bearing on the time course of the averaged pH responses. Emissions induced by 490-nm light ( $\Delta F_{490}$ ) or 440-nm light ( $\Delta F_{440}$ ) were averaged in two separate series of identical stimulus trains. The pH microelectrode confirmed the persistence of the evoked alkaline transients throughout the experiments. A semiquantitative indication of the magnitude of the optical pH signal was provided by comparing the electrode response against the late, slow components of the 490-nm signal. Slow signals evoked by CO<sub>2</sub> or by long stimulus trains were sampled at 10 or 20 Hz. Statistics were expressed as means  $\pm$  SE.

## RESULTS

### Resolution of slow pH<sub>o</sub> shifts

Baseline pH<sub>o</sub> was 7.3–7.4 in all experiments. To establish the sensitivity of the probe to shifts in pH<sub>o</sub>, slices were briefly exposed to 100% CO<sub>2</sub> (Fig. 1*A*). The resulting acid shift recorded by the pH microelectrode was closely followed by decreased  $\Delta F_{490}$  (Fig. 1*A*, *middle*). By contrast, the  $\Delta F_{440}$  was insensitive to pH (Fig. 1*A*, *bottom*). A 2% fall in the  $\Delta F_{490}$  corresponded to a shift of roughly 0.1 unit pH.

Stimulation of the Schaffer collaterals at 10 Hz (Fig. 1*B*) evoked a typical alkaline response on the pH microelectrode. This was accompanied by a more rapid increase in the  $\Delta F_{490}$  followed by a slow decay phase that closely matched the pH<sub>o</sub> recovery. There were no comparable changes in the

$\Delta F_{440}$  associated with the onset or termination of the stimulus. Similar results were obtained in three slices.

### Resolution of rapid pH<sub>o</sub> shifts

To maximize the amplitude of the alkaline shift at short latency, three supramaximal shocks were delivered at 100 Hz. This was repeated at 30-s intervals, and the fluorescence signals were averaged from 50 trains, requiring  $\sim 25$  min of recording. In the example shown in Fig. 2*A*, the  $\Delta F_{490}$  increased by  $\sim 2\%$  within 200 ms, then returned to baseline over 4 s. The same paradigm caused no detectable change in the  $\Delta F_{440}$  (Fig. 2*B*) ( $n = 6$  slices).

Figure 2*C* compares an averaged  $\Delta F_{490}$  signal against a representative pH microelectrode response. Superimposed on the optical trace (*left*) and pH electrode recording (*right*) are average fluorescence changes calculated for successive 100-ms epochs (white circles). Within the first 100-ms interval after the onset of stimulation, the fluorescence change was nearly maximal. By contrast, the pH microelectrode response was lost in a slow stimulus artifact (arrow) for 400 ms and registered a peak alkalization after nearly 1 s. However, it is notable that the later, slow signal components were recorded comparably by both methods.

The rise time of the  $\Delta F_{490}$  signal was estimated as the interval between the first stimulus and the attainment of the average fluorescence for the subsequent 200 ms. Estimates were rounded to the nearest 5 ms. In an example shown in Fig. 3*A*, the full time course of the averaged response is shown in the *top trace*. An expanded time base (Fig. 3*A*, *bottom trace*) demonstrates the rise of the signal in  $\sim 20$  ms. The peak in this example corresponded to a pH microelectrode response of +0.03 unit pH (not shown). In six slices, the estimated rise time ranged from 20 to 200 ms, averaging  $69 \pm 24$  ms. The averaged fluorescence increase was  $0.75 \pm 0.22\%$ , associated with a microelectrode response of  $+0.06 \pm 0.02$  unit pH.

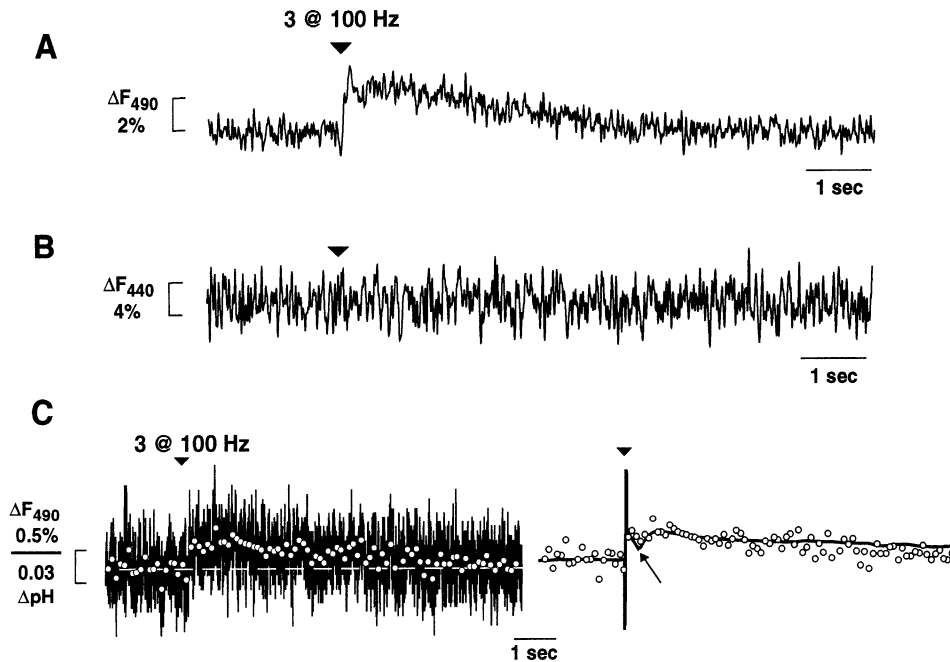


FIG. 2. Detection of rapid pH shifts evoked by a brief train. *A*: stimulation of the Schaffer collaterals (3 shocks, 100 Hz) generated a rapid increase in the  $\Delta F_{490}$ . *B*: subsequent identical stimuli produced no change in the averaged  $\Delta F_{440}$  (same slice). *C*: comparison of optical vs. electrode response. *Left*: averaged  $\Delta F_{490}$ . *Right*: pH microelectrode response from a single train. White circles: fluorescence averaged over 100-ms intervals. Arrow: slow electrical artifact. Sampling frequency was 250 Hz in *A* and *B* and 500 Hz in *C*. Optical records are averages of 50 traces.

Data from two slices with rapid responses were averaged to improve resolution (Fig. 3*B*). The three shocks evoked an average  $\Delta F_{490}$  of 0.5%, which corresponded to a microelectrode response of +0.04 unit pH. An expanded time base (Fig. 3*B*, *bottom*) demonstrates that the increase in fluorescence began before the second stimulus and was nearly fully developed within 10–20 ms.

In most experiments benzolamide was required to attain alkaline shifts with amplitudes sufficient for optical resolution (Gottfried and Chesler 1994). In two slices, particularly

robust responses were noted in the absence of the carbonic anhydrase inhibitor. In these instances the  $\Delta F_{490}$  averages displayed rise times of  $\sim 30$  and 60 ms. Figure 3*C* shows an expanded record from the former case, in which the fluorescence increased by roughly 2%, corresponding to a shift on the order of 0.1 unit pH.

#### DISCUSSION

In this report, a 2,000,000-MW fluorescein-tagged dextran was used to resolve the onset of synaptically induced pH

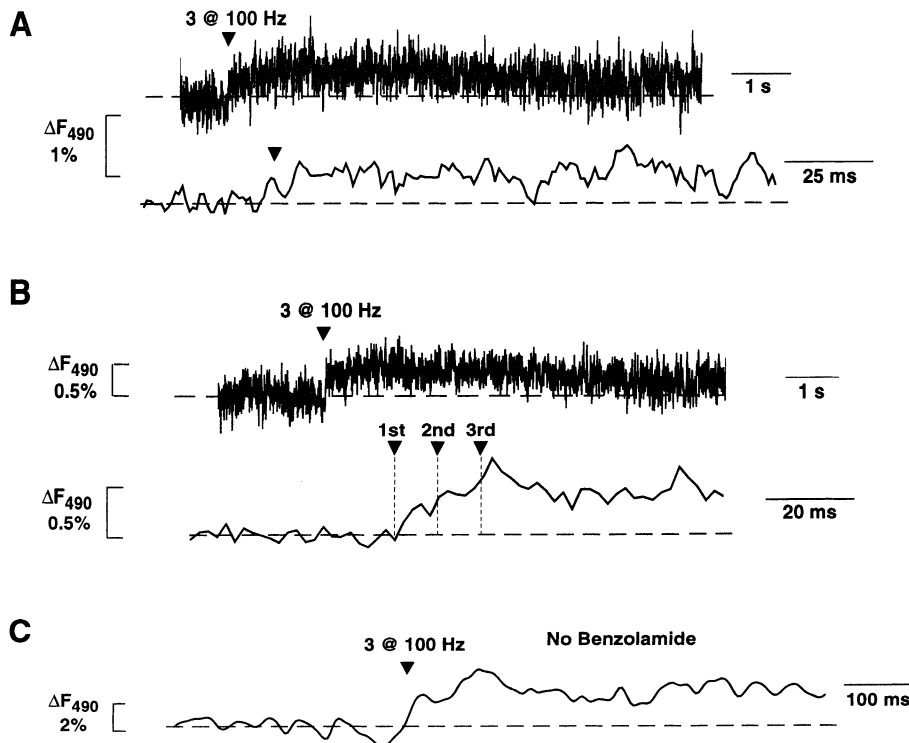


FIG. 3. Rise time of the alkaline shift in response to 3 stimuli at 100 Hz. *A*: rise of fluorescence shown at compressed and expanded time base. *B*: total of 110 responses averaged from 2 slices. Increase in fluorescence is apparent after the 1st stimulus and is well developed within 20 ms. *C*: rapid onset of alkaline shift in the absence of benzolamide. Traces in *A* and *C* were averaged from 50 trains. Sampling frequency was 500 Hz for *A*–*C*.

shifts. To our knowledge, this is the first use of such a probe for the detection of  $pH_o$  shifts evoked by neural activity. The pH-sensitive  $\Delta F_{490}$  signals were well correlated with simultaneous pH microelectrode recordings. By contrast, no stimulus-evoked changes were noted in the  $\Delta F_{440}$ . Therefore the increase in the  $\Delta F_{490}$  could not have been caused by shrinkage of the extracellular volume. The correspondence between the slow component of the optical signals and the pH microelectrode responses permitted a rough calibration. However, compared with the microelectrode recordings, fluorescence was sampled from a larger area within the depth of field. In addition, the baseline fluorescence slowly decayed. Therefore calibrations based on the pH microelectrode records should be regarded as approximate.

In a previous study by Krishtal and colleagues (1987), apparent  $pH_o$  shifts were detected by phenol red after Schaffer collateral stimulation. At room temperature, with normal extracellular  $Mg^{2+}$ , early alkalinizations were noted that required  $\sim 150$  ms to peak. The present data, obtained at  $32^\circ C$ , indicate that the activity-induced alkaline shifts can arise within 20 ms, an order of magnitude faster than previously noted. The faster onset of the alkaline shift in the present study may have been due to the higher temperature, the presence of picrotoxin, or the absence of  $Mg^{2+}$  in the superfusate. How these individual factors contributed to the speed and size of the responses awaits further study.

The onset and rise time of the alkaline shift have significance in the context of excitatory synaptic transmission. On the basis of buffering effects, an earlier study suggested that these pH changes were capable of augmenting synaptic currents mediated by NMDA receptors (Gottfried and Chesler 1994). The present results demonstrate that the onset of the alkaline transient is rapid enough to perform such modulation. Alkaline responses developed within 20 ms and had a mean rise time of 69 ms. Given an alkalinization of 0.10–0.20 unit pH, previous studies suggest that NMDA-receptor-mediated currents could be augmented by 5–10%. However, two critical points arise in this context. First, it is important to note that our optical recording method cannot determine the magnitude of  $pH_o$  shifts in the immediate proximity of postsynaptic receptors, where the modulation of synaptic currents has been inferred (Gottfried and Chesler 1994). Second, the sensitivity of the NMDA receptor to changes in pH has only been studied under conditions of agonist-receptor equilibrium (Tang et al. 1990; Traynelis and Cull-Candy 1990; Vyklícky et al. 1990). It is still unclear how the channel responds to  $pH_o$  after agonist has left the synaptic cleft.

The speed and apparent magnitude of the stimulus-evoked alkalinization suggests that the underlying process is capable of transporting a considerable acid load in a very short period. Evidence from recent studies has suggested that plasmalemmal  $Ca^{2+}$ - $H^+$  exchange could be responsible for these pH changes (Paalasmaa et al. 1994; Schwiening et al. 1993; Smith et al. 1994). Whether this process can account for both the speed and magnitude of the response remains to be determined.

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