Published in final edited form as:

J Neurogenet. 2011 October ; 25(3): 104–114. doi:10.3109/01677063.2011.606577.

Extracellular Protons Reduce Quantal Content and Prolong Synaptic Currents at the *Drosophila* Larval Neuromuscular Junction

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Abstract

Fluctuations in extracellular pH occur in the nervous system in response to a number of physiological and pathological processes, such as ischemia, hypercapnea, and high-frequency activity. Using the *Drosophila* larval neuromuscular junction, I have examined acute effects of low and high pH on excitability and synaptic transmission. Acidification rapidly and reversibly reduces the size of electrically evoked excitatory junctional currents (EJCs) in a concentration-dependent manner, with transmission nearly abolished at pH 5.0. Conversely, raising pH to 7.8 increases EJC amplitude significantly. Further elevation to pH 8.5 causes an initial increase in amplitude, followed by profound, long-lasting depression of the synapse. Amplitudes of spontaneous miniature EJCs (mEJCs) are modestly, but significantly reduced at pH 5.0. It is therefore the number of quanta released per action potential, rather than the size of individual quanta, that is most strongly affected. Decay times of both EJCs and mEJCs are dramatically lengthened at low pH, suggesting that glutamate remains in the synaptic cleft for much longer than normal. Presynaptic excitability is also reduced, as indicated by increased latency between nerve shock and EJC onset. The response to low pH was not altered by mutations in genes encoding TRPML and Slowpoke ion channels, which had previously been implicated as possible targets of extracellular protons. I conclude that extracellular protons have strong effects on the release of glutamate and the time course of synaptic currents. These phenotypes can be exploited to study the mechanisms of acid-mediated changes in neuronal function, and to pursue the way in which pH modulates synaptic function in normal and pathophysiological conditions.

Introduction

Despite elaborate mechanisms for buffering the nervous system against pH fluctuations, extracellular acidification occurs under a variety of physiological and pathological conditions. At the extreme end of pathology, extracellular pH can drop as low as 6.0 during ischemic events, when aerobic metabolism fails and lactic acid accumulates (Tombaugh and Sapolsky, 1993). During respiratory acidosis, CO₂ in the extracellular fluid is converted to carbonic acid by carbonic anhydrase, which acts on chemosensory neurons to increase ventilatory drive (Denton et al., 2007; Putnam et al., 2004). Even during normal activity, pH can fluctuate in restricted extracellular space as a result of, for example, bicarbonate ions diffusing out of neurons through GABA receptor channels, or protons being secreted along with the contents of synaptic vesicles (Dietrich and Morad, 2010; Palmer et al., 2003).

Understanding cellular mechanisms underlying the neuronal response to protons can therefore provide significant insight into the processes of ischemic insult, chemosensation, and synaptic plasticity. The neuromuscular junction of the *Drosophila* larva is a useful model, in that it is glutamatergic, like most mammalian excitatory central synapses, and

provides an impressive toolkit of electrophysiologic, genetic and transgenic methods that enable detailed analysis of pre- and postsynaptic function. Earlier work using current clamp recording showed that the larval neuromuscular junction is sensitive to both protons and CO₂ (Badre et al., 2005), in that pH 5.0 inhibited synaptic transmission, and CO₂ appeared to abolish the response to glutamate.

The present study uses voltage clamp, which allows more precise measurement of the amplitudes and kinetics of evoked and quantal currents, to examine the effects of pH across more than three orders of magnitude of proton concentration (pH 5.0 to 8.5). Low extracellular pH rapidly reduced evoked synaptic currents (excitatory junctional currents, EJCs) in a concentration-dependent way. Sizes of spontaneous miniature EJCs (mEJCs) were reduced only slightly at the lowest pH, indicating that the dominant effect was on neurotransmitter release. EJCs were potentiated by elevated pH, but slowly-developing, long-lasting depression occurred at pH 8.5, the highest pH tested. At low pH, there was a several-fold slowing of decay time of synaptic currents, which may indicate prolonged residence of glutamate in the synaptic cleft. The latency between stimulus and arrival of EJCs became longer, consistent with decreased presynaptic excitability. Therefore, extracellular protons affect specific axonal and synaptic functions at the larval neuromuscular junction, providing novel insight into the neural response to acidification.

Methods

Husbandry and Strains

Flies were cultured in vials on standard cornmeal molasses diet at 25°C. Except when otherwise specified, the wild-type strain, *Canton-S*, was used for all experiments. *trpm1^l*, *p[trpm1];trpm1^l* and a deficiency for the region (*Df(3L)exel6135*) were obtained from the Bloomington Stock Center, and *trpm1^{c05523}* came from Harvard. *trpm1* alleles were in a *white¹¹¹⁸* (Exelixis) background, balanced over *TM6*, marked with *Tubby* and *Humeral*. *slowpoke^l* (*slo^l*), marked with *scarlet*, was a generous gift from Dr Nigel Atkinson (University of Texas, Austin). The congenic *w¹¹¹⁸* control strain for the mutants was generated via removal of two Exelixis *piggyBac* insertions by recombination. To reduce function maximally, and avoid possible complications from unrelated polymorphisms, *trpm1* alleles were tested in combination with the deficiency.

Electrophysiology

Excitatory junctional currents (EJCs) and miniature EJCs (mEJCs) were recorded in two-electrode voltage clamp as described previously (Sandstrom, 2004). Recordings were performed in HL3 saline (in mM: 70 NaCl, 5 KCl, 1 CaCl₂, 20 MgCl₂, 10 NaHCO₃, 5 trehalose, 115 sucrose, 5 HEPES; (Stewart et al., 1994). pH was adjusted with 1 M HCl or 1 M NaOH, as appropriate. Osmolality (~310 mOsm; Vapro 5520, Wescor, Logan UT) was not measurably affected by the small amount of HCl required to adjust pH.

A subset of experiments was performed in saline with 4 mM Mg²⁺ ("HL3.1"; Feng et al., 2004). Reduction of Mg²⁺ caused a 3- to 4-fold increase in quantal content of evoked release, which necessitated reducing Ca²⁺ to 0.3 mM in order to maintain release in a relatively linear range. Under these conditions, sensitivity to pH was enhanced, so that release was nearly eliminated in wild-type controls at pH 5.75. Elevating experimental pH slightly to 6.1 elicited a strong reduction in synaptic currents, but EJCs remained large enough to reveal possible enhancement or suppression by the mutants. Therefore pH 6.1 was used to test *trpm1^{c05523}* and *slo^l* mutants.

Muscle 6 in segment A5 was voltage clamped at -70 mV with an Axoclamp 2B, controlled by pClamp version 8.2 or 9.2 (Sandstrom, 2004). Solutions were selected using a solenoid

valve controlled by pClamp, and perfused with a peristaltic pump (Harvard Apparatus, Holliston, MA). EJCs were evoked by shocking the cut end of the nerve innervating the muscle every 10 s. Amplitudes, rise times, and decay times of synaptic currents were measured in MiniAnalysis (Synptosoft, Decatur GA). Latency was measured as the time from the start of the stimulus artifact to the onset of the EJC. For statistical comparison and bar graphs, the averages of 10 EJCs before, during and after treatment were used for each preparation. Apparent failures were scored as amplitude = zero. mEJC parameters were determined from continuous 25 minute recordings of unstimulated preparations. Amplitudes were determined by averaging the sizes of events occurring 100 s before, during and after treatment. Average mEJC size was determined by fitting mEJC amplitude distributions with Gaussians in Origin 5.0 (Fatt and Katz, 1952). Rise and decay times of mEJCs were measured from signal-averaged mEJCs from these same time periods. All electrophysiology traces and graphs were plotted in Origin.

Statistical analysis was performed in Sigmaplot 11.0. Data that fit the assumptions for parametric statistics were compared using one-way ANOVA. Data showing significant skew or heteroskedacity were analyzed using the Kruskal-Wallis test. Numbers presented are means $\pm$ standard error of the mean. For estimated quantal content, standard errors were calculated by using the sum of the squares of the relative errors of EJC and mEJC amplitudes, to generate a relative error for quantal content (Meyer, 1975).

Results

Low pH Reduces Synaptic Strength

In the process of examining the pH sensitivity of an inhibitory conductance activated by the volatile anesthetic isoflurane (Sandstrom, 2008), I found that reducing the pH of the recording solution to 6.25, which should maximally inhibit TASK channels (Döring et al., 2006), reversibly reduced the size of excitatory junctional currents (EJCs; Figs. 1A, 1B). Reduction in amplitude was accompanied by pronounced slowing of EJC decay (Fig. 1A). This response did not resemble the effect of inhibiting TASK channels, and did not alter the effects of the volatile anesthetic isoflurane (Sandstrom, 2008), but the rapid, robust and reversible inhibition of synaptic transmission warranted further investigation.

Low pH acted in a concentration-dependent manner, with EJC amplitude decreasing as the pH dropped (Figs. 1B, 1C). While lowering pH from the standard 7.1 to 6.75 did not alter EJC amplitude significantly, pH 6.25 had a modest but significant effect on EJC size (one-way ANOVA, $p < 0.01$), and an additional drop of 0.5 units to 5.75 reduced amplitude by more than 60%. Evoked synaptic currents were still detectable at pH 5.0, although there were many apparent failures. Because failures only occurred in the subset of experiments at pH 5.0 in which EJC amplitude approached zero, and under no other conditions, it is likely that failure is due to disruption of excitation-secretion coupling rather than action potential block.

As can be seen in Figure 1B, the effect on EJC amplitude was relatively rapid, reaching a minimum within a few minutes of solutions being switched, at least one minute of which was required for solutions to exchange. EJC amplitude remained depressed as long as the preparation was superfused with acidic solution. When the preparation was returned to pH 7.1, EJC amplitude rapidly returned to control levels. EJC amplitudes in low pH-treated preparations rebounded significantly above those in untreated controls (Fig. 1B, inset; $p < 0.05$, one-way ANOVA), presumably because reduction of EJC amplitude by acidification prevented low-frequency depression (Sandstrom, 2004; Wu et al., 2005).

While modestly increasing pH to 7.8 had the opposite effect of acidification, increasing EJC amplitude significantly (Fig 1B, 1C; $p < 0.01$, one-way ANOVA), further increase to pH 8.5 caused more complex effects. An initial increase in amplitude was followed by a gradual and long-lasting depression (Fig 1D, 1E). During the wash, EJC amplitude decreased rapidly, as if acute, alkaline-induced facilitation was removed and the full effects of accumulated synaptic depression were revealed. For the remainder of the experiment, EJC amplitude remained depressed, but steady.

Thus, between pH 5.0 and 7.8, a range of nearly three orders of magnitude of $[H^+]$, there is an almost linear relationship between pH and EJC amplitude (Figure 1C). Below pH 5.0, transmission fails, and above pH 7.8 the synapse is subject to long-lasting depression.

pH Affects EJC Kinetics

EJC shape also changes in response to pH. Compared to controls, EJC decay time was slower at low pH (Fig. 1A) and faster at high pH (Fig. 1D). Decay time constants (τ_{decay}) for signal averaged EJCs were most strongly affected by low pH, although elevated pH caused significant acceleration of EJC decay (Figs 1D, 2A; $p < 0.05$, Kruskal-Wallis). Below pH 6.25, τ_{decay} increased dramatically and significantly, with average values more than doubling at pH 5.75 and 5.0. As discussed in more detail below, this effect could result from loss of synchrony of release, or from changes in the time courses of the quantal events that make up the evoked EJCs.

In addition to slowing the decay of the EJC, low pH also slowed its rise to peak amplitude (Fig 2B, 2C). At pH 5.75, there was a subtle and variable reduction in the slope of the EJC (Fig. 2B, grey trace) that resulted in a statistically significant increase in the time required for the EJC to rise from 10% to 90% maximum amplitude (Fig. 2C; $p < 0.05$, Kruskal-Wallis, $n = 7$).

Low pH Alters mEJC Size and Shape

To examine the effects of low pH on the size and shape of quantal events, continuous records of spontaneous mEJCs were collected in unstimulated preparations under the same conditions as above. At pH 5.0, evoked release was nearly abolished, but spontaneous mEJCs persisted (Fig 3A). Signal-averaged mEJCs revealed a small reduction in amplitude and a pronounced prolongation of decay (Fig 3B). Quantification of amplitude distributions showed a ~22% reduction in mEJC amplitude, which was statistically significant (Fig 3C; $p < 0.01$, one-way ANOVA, $n = 966$ events before, 584 events during, 588 events after treatment, in 5 preparations), and presumably contributed to the reduction of EJC amplitude. However, when average EJC amplitude is divided by mean mEJC amplitude to provide an estimate of quantal content, the number of vesicles released is reduced by more than 90% (Fig. 3D).

Low pH produced a several-fold increase in decay time of mEJCs (Fig. 3B, 3E). At pH 7.1, the decay time constant (τ_{decay}) for signal-averaged mEJCs was 6.15 ± 0.4 ms ($n = 5$), similar to values reported previously (Heckmann and Dudel, 1998). At pH 5.0, τ_{decay} increased threefold, to an average of 18.0 ± 1.9 . These results confirm that the slowing of τ_{decay} of evoked EJCs is largely or entirely due to effects on quantal events. Rise times of mEJCs were not significantly affected by pH (mean time 10–90% maximum amplitude (ms) pH 7.1 pre, 1.09 ± 0.05 ; pH 5.0, 1.26 ± 0.08 ; pH 7.1 wash, 1.20 ± 0.10 ; $p > 0.05$, one-way ANOVA, $n = 5$).

At pH 5.0, mEJC frequency decreased modestly, but not significantly (Fig. 3F). mEJC frequency decreased to approximately 60% of control (pH 7.1 pre, 1.93 ± 0.34 mEJC s^{-1} ; pH 5.0 1.17 ± 0.20 ; pH 7.1 wash, 1.18 ± 0.10). The effect was variable, and although

ANOVA identified a significant effect overall, post-hoc tests did not demonstrate significance ($p > 0.05$, Holm-Sidak). These data do not rule out a possible effect of low pH on secretion, but the magnitude of this change is small compared to the reduction of EJC size (Fig. 1B, 1C) or estimated quantal content (Fig 3D), indicating that other mechanisms are more important.

Low pH reduces Presynaptic Excitability

As shown above, the inhibitory effect of low pH on synaptic transmission was primarily presynaptic. Reduced glutamate release was associated with an increase in latency from nerve shock to the onset of the EJC, adding 0.5–1.0 ms to the time required for it to arrive at pH 5.75 (Fig 4A, inset). This effect occurred rapidly, and reversed upon return to pH 7.1 (Fig. 4A). Increasing pH to 7.8 may have reduced latency somewhat (Fig. 4A), but a further increase to pH 8.5 resulted in a slow developing long-lasting increase in latency (Fig. 4B).

As with EJC decay, the concentration-response relationship showed that the strongest effects on latency occurred at low pH (Fig. 4C). The effect of pH 7.8 was not statistically significant, while pH 5.75 to pH 5.0 progressively increased latency ($p < 0.05$, Kruskal-Wallis).

The increase in time from stimulus to EJC presumably resulted from an increase in the time for the axon to reach spike threshold, a decrease in axonal conduction velocity, or both (Sandstrom, 2004). Based on increased EJC rise time (Fig. 3), excitation-secretion coupling also appears to be affected, but would not be expected to change latency. Instead, the effect is likely to be mediated by one or more ion channels.

Ion Channel Mutants

Although the list of possible channels targeted by pH is long, I selected the Slowpoke Ca^{2+} -activated K^+ and TRPML channels as initial candidates.

The TRPML channel was identified as possibly pH-sensitive based on the CO_2 hypersensitivity exhibited by mutants (Venkatachalam et al., 2008). To analyze the effects of *trpml* on pH response, I used *trpml^l*, a previously described null allele (Venkatachalam et al., 2008) and *trpml^{lc05523}*, an allele generated by insertion of a *piggyBAC* transposon in the second intron. Based on its semi-lethality in combination with *trpml^l* and a chromosomal deficiency for the region, *trpml^{lc05523}* is a bona fide *trpml* allele: 20.3% of *trpml^{lc05523}/Df* males emerged compared to 4% of *trpml^l/Df* males; females of both genotypes failed to emerge. *trpml^l/trpml^{lc05523}* had higher viability, with 61.2% of males and 8.2% of females emerging. *trpml^{lc05523}* also failed to complement *trpml^l* or the deficiency for a previously unreported crumpled wing phenotype (Fig 5A). Among the few mutant flies that emerged ($n = 18$) the phenotype was 100% penetrant. Importantly, the wings were rescued by a single copy of a transgene containing *trpml* genomic DNA (Fig. 5A; $n = 29$). Curiously, despite having clear effects on viability and wing structure, neither allele showed evidence of reduced EJC amplitude previously reported (Fig 5B; (Venkatachalam et al., 2008)).

As described above, pH 5.75 strongly reduces but does not eliminate EJCs, providing a background against which enhancement or suppression can be measured. When *trpml^l/Df* mutants were challenged with pH 5.75, their responses did not differ from congenic controls (Fig 5C).

I also tested *trpml* mutants in HL3 saline in which Mg^{2+} was reduced from 20 to 4 mM (“HL3.1”), which can enhance the effects of some mutants on excitability and synaptic transmission (Feng et al., 2004). Because reduction of Mg^{2+} altered baseline synaptic transmission and enhances the response to acidic pH (see Methods), Ca^{2+} was reduced to 0.3

mM, and the experimental pH was raised from 5.75 to 6.1. As was the case with 20 mM Mg^{2+} , neither baseline EJC amplitude of *trpm1* mutants (Fig. 5D) nor their response to low pH (Fig. 5E) was different from the wildtype. I could therefore find no evidence that the TRPML channel played a role in the response to low pH.

Slowpoke (Slo) is the *Drosophila* ortholog of the large-conductance Ca^{2+} -activated K^{+} channel (BK). In vertebrates, BK channels are inhibited by acid-sensing ion channels (ASICs), through a direct physical interaction which is relieved by extracellular acidification (Petroff et al., 2008). Although the expression of ASIC channels at the neuromuscular junction is not yet described, the role of *slo* is well established at this synapse (Warbington et al., 1996; Zhou et al., 1999) suggesting that activation of Slo by extracellular protons could mediate the effects of low pH. Reducing *slo* function might therefore suppress these effects. Although previously reported *slo¹* mutant phenotypes, such as reduced EJC amplitude (Fig 5D; Warbington et al., 1996), and the tendency to fire repetitively upon stimulation (data not shown), were clearly present, the response of *slo¹* mutants to pH 6.1 did not differ from those of the wildtype (Fig 5E).

Discussion

Low pH affected neurotransmission rapidly and reversibly, changing the sizes and shapes of quantal events and reducing the number of quanta released. EJCs became smaller, rose and decayed more slowly, and arrived at the synapse with a longer delay after the stimulus. Despite pH 5.0 being an extreme environment, the relatively narrow range of effects, the similarity of their concentration response relationships and time courses imply the presence of only a few molecular targets.

Altered mEJC Amplitude and Kinetics

All major subclasses of mammalian glutamate receptors are modulated by protons (Ihle and Patneau, 2000; Mott et al., 2003; Traynelis and Cull-Candy, 1991), with most being inhibited. Therefore, it may not be surprising that mEJC amplitude decreases at low pH. Based on the changes in mEJC kinetics, however, decreased glutamate receptor sensitivity is not the entire story.

The most obvious effect of low pH on mEJCs was the several-fold increase in the decay time constant (τ_{decay}). At pH 7.1, τ_{decay} is 6.15 ms, similar to previously reported values, and too fast for desensitization to play a significant part (DiAntonio et al., 1999; Heckmann and Dudel, 1997), while at pH 5.0, τ_{decay} increases to 18.0 ms, a value identical to that reported for glutamate receptor desensitization (DiAntonio et al., 1999). These results suggest that glutamate remains in the synaptic cleft long enough for desensitization to determine the decay kinetics of synaptic currents.

Jan and Jan (1976) observed a similar prolongation of synaptic current decay in the presence of exogenous L-glutamate, and suggested that the additional glutamate saturated transporters at the synapse. Although the formal possibility exists that increases in τ_{decay} are due to inhibition of transporters by protons or glutamate, there are currently no transporters known that actively remove glutamate from the larval synapse (Rival et al., 2006). Therefore, the most likely mechanism underlying the slowed decay of synaptic currents is prolongation of glutamate release. In the absence of inflections in mEJCs that would suggest multi-quantal events, a possible mechanism for prolongation of glutamate release is suggested by the observation that the shapes of mEJCs appear to depend on the dynamics of the fusion pore itself (Pawlu et al., 2004). If low pH resulted in further slowing of vesicular glutamate release, glutamate would remain longer in the cleft, with the additional consequences of increased mEJC rise time and reduced peak amplitude. In principle, therefore, all of the

observed effects on mEJC amplitude and kinetics could result from changes in vesicle fusion.

Presynaptic Excitability and Evoked Release

The modest effects on mEJC amplitude indicate that low pH reduces EJC amplitude by reducing quantal content, *i.e.*, the number of vesicles released. The potentially large number of possible target molecules includes ion channels, the vesicle fusion complex, presynaptic neurotransmitter receptors, or second messenger systems that affect one or more of these.

The slowing of the rate of rise of the EJC suggests that excitation-fusion coupling is perturbed. Molecules involved in vesicle fusion, such as synaptotagmin and SNAREs are in fact of potential interest because of the hypothesized role of the fusion pore (Almanza et al., 2008; Vessey et al., 2005). However, increased EJC latency implies additional targets in the axon. Altered ion channel function could, for instance, result in slower action potentials that produce weaker depolarization of the synaptic terminal, thereby increasing latency and reducing EJC amplitude (Sandstrom, 2008).

The number of ion channels known to be modulated by pH is growing rapidly, and includes, among others, voltage-gated ion channels, many types of TRP channels, acid-sensing ion channels (ASICs), tandem-pore K⁺ channels, and ionotropic neurotransmitter receptors (reviewed in Holzer, 2009). The effects of many of the known acid-regulated channels, such as ASICs and tandem pore channels, are inconsistent with the effects observed in the present study, causing excitation rather than inhibition. Others, such as GABA_A receptors, are not found at the larval neuromuscular junction (Featherstone et al., 2000). Inhibition of the voltage-gated Na⁺ channel encoded by the *paralytic (para)* gene would have the observed effect of low pH, and it is present in the presynaptic axon, but its role in the response to pH would be difficult to test. Previous work has shown that reduction of *para* function will enhance the effects of any manipulation that reduces excitability, regardless of mechanism (Sandstrom, 2008), and there are currently no tools for reliably increasing *para* function *in vivo*.

Slo and TRPML channels were plausible targets, and mutants were predicted to suppress the effects of low pH. However, it appears that neither strong hypomorphic mutants (*slo*¹ and *trpml*^{K05523}) nor a null mutant (*trpml*¹) affected the synaptic response to low pH. Many more channels remain to be tested.

My failure to replicate the reduction of baseline synaptic transmission by *trpml* mutations (Venkatachalam et al., 2008) could result from a number of causes. It is possible that the *w*¹¹¹⁸ strain that I used, which is congenic with *trpml*^{K05523} and *Df(3L)exel6135*, differs from the *w*¹¹¹⁸ used by Venkatachalam *et al.* The disparity may also have resulted from differences in the physiological salines used, or in rearing conditions.

The enlargement and narrowing of the EJC at pH 7.8 appears to be a simple reversal of the reaction to low pH, and presumably involves the same target(s). Long-lasting inhibition of the synapse at pH 8.5 is reminiscent of activation of an inhibitory conductance, such as K_{2p} or K_{IR}. The slow onset of the effect and apparent lack of reversal suggest the occurrence of possible posttranslational modifications, or perhaps alteration of protein structure resulting from unnaturally high pH.

Acidosis, Pathology and Neuromodulation

Acidification is relevant to the nervous system under a number of circumstances, such as ischemia, hypercapnea, and CO₂ anesthesia. Furthermore, as discussed below, there is

growing evidence that protons regulate synaptic transmission under normal physiological conditions.

Acidification plays a central role in the effects of cerebral ischemia, one of the leading causes of neuropathology. When aerobic metabolism fails during an ischemic event, metabolic acids build up in the interstitial space, while ionic gradients break down, and extracellular glutamate levels rise (Hertz, 2008). These conditions can lead to neuronal depolarization and excitotoxicity. On balance, the effects described here would be neuroprotective. Reduced neuronal excitability and synaptic transmission would reduce the metabolic load, slow the breakdown of ionic gradients, and reduce the amount of glutamate released into the extracellular space. This is similar to the neuroprotective responses to acidosis in the mammalian brain (Tombaugh and Sapolsky, 1993).

Anesthesia by CO₂ is well documented in mammals (Brosnan et al., 2007; Eisele et al., 1967) and its use with *Drosophila* is nearly ubiquitous (Greenspan, 2004). *Drosophila* biologists generally use saturating levels of atmospheric CO₂, which are expected to decrease pH of the hemolymph to approximately 5.0 (Badre et al., 2005). The severe disruption of synaptic transmission by low pH, as described here, would most certainly contribute to a state of immobilization.

Extracellular pH is also relevant to synaptic transmission under more physiological conditions. Depending on the neurons and the context, neuronal activity can acidify or alkalize the surrounding fluid (Shuba et al., 2008; Tong et al., 2006). For example, the internal pH of synaptic vesicles is strongly acidic (Miesenböck et al., 1998). Release of these protons during exocytosis can therefore measurably reduce extracellular pH and reduce subsequent release via inhibition of voltage-gated Ca²⁺ channels (Palmer et al., 2003; Shuba et al., 2008).

As might be expected, the effects of pH on neuronal function are best observed in the absence of strong, synthetic buffers, such as HEPES (Dietrich and Morad, 2010), which are components of all routinely used *Drosophila* physiological salines (Feng et al., 2004; Stewart et al., 1994). Larval motoneurons produce high frequency bursts of action potentials during fictive locomotion (*e.g.*, Barclay et al., 2002), which rapidly release the contents of hundreds or thousands of vesicles over a time course of milliseconds. This suggests the intriguing possibility that in the extracellular fluid of the intact animal, pH-mediated alterations in quantal size, quantal content, and kinetics would be superimposed upon previously described forms of short-term plasticity (Broadie et al., 1997; Zhong and Wu, 1991).

Acknowledgments

This research was supported by the Intramural Program of NIMH, NIH. Thanks to Dr. Howard Nash (NIMH) for space, material and intellectual support, and Dr. Benjamin White (NIMH) for advice and the use of equipment. Thanks to Drs. Howard Nash, Subhabrata Sanyal, and Elizabeth Sherman for critical comments on this manuscript. Mutant fly strains were generously provided by the Bloomington Stock Center and Dr. Nigel Atkinson (University of Texas, Austin).

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Figure 1.

pH alters EJC amplitude. A. Traces from a representative experiment, in which pH 6.25 reduced EJC amplitude (gray trace) compared to those at pH 7.1 before and after (black traces). Decay time also increased. Scale bar: vertical 20 nA, horizontal 10 ms. B. Time course of pH effect on EJC amplitude. Each point is normalized to the average of the first ten responses for each treatment (mean $\pm$ s.e.m.). In untreated controls (pH 7.1, filled squares), synapse strength remained stable, with slight low-frequency depression. When perfused with low pH solutions, the synapse responded within two minutes, at least one minute of which is required for solution exchange. EJCs in acid treated preparations rebounded significantly above control amplitude upon return to pH 7.1 (inset; pH 5.75 shown; $p < 0.05$, one-way ANOVA). C. Relationship between pH and EJC amplitude. Each point represents EJC amplitude at minutes 11–13 (mean $\pm$ s.e.m.), normalized to the average of the first 10 responses for each treatment. Decreasing pH to 6.75 had no significant effect, whereas at pH 6.25, amplitude is significantly different from pH 7.1 (asterisk). At pH 5.0, EJCs are measurable, but interspersed with many failures to detect transmission. Raising pH to 7.8 significantly increased EJC amplitude. Sample sizes are shown in parentheses below each symbol. D. Representative traces, showing transient increase in EJC amplitude, with more rapid decay, at pH 8.5 (grey trace), followed by depression (wash). Scale bar: vertical 10 nA, horizontal 10 ms. E. Time course of response to pH 8.5. Compared with untreated controls (black squares), EJCs in preparations subjected to pH 8.5 (open circles) transiently

became larger, then slowly declined (mean $\pm$ s.e.m.). After return to pH 7.1, EJC amplitude reached a minimum that remained steady for at least 10 minutes.

Figure 2.

Effects of pH on EJC kinetics. A. Relationship between pH and decay time constant (τ_{decay} ; mean $\pm$ s.e.m.). For each sample, 10 traces were signal averaged from $t = 11-13$ min and fitted with a single exponential ($= \tau_{\text{decay}}$). This τ_{decay} was then normalized to that from the first 10 traces collected at $t = 0-2$ min in the same preparation. pH 5.0, 5.75, 6.25, 7.8 and 8.5 differed significantly from pH 7.1 ($p < 0.05$, Kruskal-Wallis). B. Traces from control (black trace) and pH 5.75 (grey trace), aligned at onset of EJC and rescaled in the Y axis (current) to compare slopes. Each is an average of 10 traces, scale = 1 ms. C. The time for the EJC amplitude to rise from 10% to 90% maximum amplitude is significantly increased at pH 5.75 (mean $\pm$ s.e.m.; Kruskal-Wallis, $p < 0.01$, $n = 7$).

Figure 3.

Effects of low pH on miniature EJCs (mEJCs). A. mEJCs in control (pH 7.1, top trace), pH 5.0 (middle trace) and after washing with pH 7.1 (bottom trace). Amplitude and frequency were not drastically affected. Scale bars: vertical 1.0 nA, horizontal 500 ms. B. Averaged mEJCs from a representative preparation, showing a slight reduction in amplitude (arrow) and marked increase in decay time at pH 5.0 (grey trace). Scale bars: vertical 0.2 nA, horizontal, 20 ms. C. Average mEJC size decreases by approximately 22% in pH 5.0 (mean $\pm$ s.e.m.; $p < 0.01$, $n = 5$ preparations). D. Quantal content, calculated as the average EJC amplitude divided by the average mEJC amplitude (mean $\pm$ s.e.m.). Because EJCs and mEJCs were collected in separate experiments, standard errors were calculated by error propagation. E. mEJC decay at pH 5.0 (mean $\pm$ s.e.m.), determined by fitting single time constants to averaged records such as that shown in panel B. Decay time increases many-fold and significantly ($p < 0.05$, Kruskal-Wallis). F. mEJC frequency, measured in 60s bins from continuous 25 min records. mEJC frequency appeared to decrease in response to pH 5.0, but the effect was not statistically significant (mean $\pm$ s.e.m.; $p > 0.05$, one-way ANOVA).

Figure 4.

pH alters presynaptic excitability. A. Timecourse of change in latency in response to pH 5.75 and 7.1. Latency (mean $\pm$ s.e.m.), defined as the time from the onset of the stimulus artifact to that of the EJC, increases in response to lowered pH, with a similar time course to the reduction in amplitude (see Fig 1B). The effect reverses upon return to pH 7.1. Inset shows EJCs recorded in pH 7.1 (black trace) and pH 5.75 (grey trace), with arrows indicating the time of onsets of the two EJCs. Note that, although the EJC amplitude at pH 5.0 is small, the time of onset is distinct and easily measured. Scale bar: 1 ms horizontal, 20 nA vertical. B. In response to pH 8.5, there was little initial change in latency followed by a steady increase (mean $\pm$ s.e.m.). Latency reached a peak after return to pH 7.1, and remained steady thereafter. C. Concentration response. Latency (mean $\pm$ s.e.m.) is not significantly affected between pH 7.8 to 6.25, but is significantly longer at pH 5.75 and below.

Figure 5.

Effect of *trpm1* and *slowpoke* mutants on low pH response. A. *trpm1* alleles fail to complement one another for crumpled wing phenotype. Left two panels show the uninflated wings of *trpm1^{c05523}/Df* and *trpm1^{c05523}/trpm1^l*. Right panel shows complete rescue of wing phenotype by a single copy of *p[trpm1]* in a *trpm1^{c05523}/trpm1^l* background. B. Baseline EJC amplitudes of *trpm1* mutants do not differ from the wildtype in HL3 saline (mean $\pm$ s.e.m.). C. *trpm1^l* neither enhances nor suppresses the effect of pH 5.75 on EJC amplitude (mean $\pm$ s.e.m.). D. Baseline EJC amplitude (mean $\pm$ s.e.m.) in *trpm1* mutants is normal in HL3.1 saline. EJC of *slo¹* mutants are significantly reduced. E. *trpm1^{c05523}* (open circles) and *slo¹* (grey triangles) mutations do not affect the magnitude or time course of the response to pH 6.1, compared to congenic controls (black squares; mean $\pm$ s.e.m.). Because absolute EJC amplitude was significantly smaller in *slo¹* mutants, amplitudes of EJC at pH 6.1 were also smaller (*slo¹* 4.4 ± 0.65 nA vs 20.1 ± 4.9 nA in the wildtype), resulting in significantly more rebound in the mutants after return to pH 7.1 (p, 0.05, one-way ANOVA).