The Effect of CO_2, Intracellular pH and Extracellular pH on Mechanosensory Proprioceptor Responses in Crayfish and Crab

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ABSTRACT

Proprioceptive neurons monitor the movements of limbs and joints to transduce the movements into electrical signals. These neurons function similarly in species from arthropods to humans. These neurons can be compromised in disease states and in adverse environmental conditions such as with changes in external and internal pH. We used two model preparations (the crayfish muscle receptor organ and a chordotonal organ in the limb of a crab) to characterize the responses of these proprioceptors to external and internal pH changes as well as raised CO_2. The results demonstrate the proprioceptive organs are not highly sensitive to changes in extracellular pH, when reduced to 5.0 from 7.4. However, if intracellular pH is decreased by exposure to propionic acid or saline containing CO_2, there is a rapid decrease in firing rate in response to joint movements. The responses recover quickly upon reintroduction of normal pH (7.4) or saline not tainted with CO_2. These basic understandings may help to address the mechanistic properties of mechanosensitive receptors in other organisms, such as muscle spindles in skeletal muscles of mammals and tactile as well as pressure (i.e., blood pressure) sensory receptors.

KEYWORDS

Proprioception; Sensory; Invertebrate; Carbon Dioxide; Protons; Mechanosensory; Intracellular pH; Extracellular pH

INTRODUCTION

Cellular metabolism in animals produces CO_2 as a by-product in the production of ATP, which is rapidly buffered or eliminated to maintain a homeostatic balance in dissolved CO_2, pH and HCO_3^-. The interrelationship between pH and CO_2 goes beyond the equilibrium of $CO_2+H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$,[3] as H^+ ions are also in balance with other buffering mechanisms within cells as well as within the extracellular fluid (ECF). Cellular composition and ECF both contribute in many ways to buffering H^+ as well as transporting CO_2 in various states (soluble and HCO_3). Each organelle and cytoplasm of cells maintains a different pH level. This demonstrates that H^+ balance is uniquely regulated by various process including pumps, exchangers, and transportors.[4] In spite of experimentally lowering extracellular pH, the cytoplasm of many cells will not match the extracellular pH.[5,6] Thus, it cannot be assumed that extracellular pH (pH_o) is in equilibrium with the pH in cells (pH_i) as protons are not freely diffusible across the cell bilipid membrane.

Increased cellular metabolism for tissues (e.g. skeletal muscle or neural tissue) can have rapid effects on the blood/hemolymph pH and is usually dealt with quickly in reaching a homeostatic level. Some organisms can withstand wide ranges in pH and CO_2 levels within the blood/hemolymph without any apparent pathological conditions while other organisms cannot. An example of this is exhibited in honeybees, which can use a defense with group effort in producing CO_2 and increasing temperature in order to kill an invading hornet as the honey bee can tolerate the acute changes in the environment.[7] Early methods in mammalian anesthesia for surgery was to have patients rebreathe exhaled air resulting in increased CO_2. This suggests an inability to withstand

[†] This course project is part of a new trend in teaching science to undergraduates.[1] Course-based undergraduate research experiences (CUREs) are relatively new and an approach being adopted by science educators in high schools and colleges.[2] The course is neurophysiology lab (Bio446, Bio650).

rapid changes in CO_2 levels.[8] It is not fully understood why some organisms are more resilient to pH/CO_2 alterations, but further elucidation in this field would benefit both general knowledge and could lead to potential therapeutics in mammalian pathological conditions (SIDS, COPD, ischemic conditions). Humans are known to display some acclimatization to altered pH/CO_2 balance with prolonged pathological conditions. It is generally acknowledged that people with chronic obstructive pulmonary disease (COPD) become less responsive to increased levels of blood pCO_2 (low pH) to drive breathing and become more driven by hypoxia (low pO_2) for respiratory control.[9-11] However, the effects on sensory neurons for proprioception in such altered conditions in humans has not been addressed as far as we are aware.

Abnormally low pH or higher CO_2 in neural tissue can occur during pathological states (brain ischemia, hypercapnia, COPD) or by intentionally manipulating CO_2 levels for research purposes.[12-14] CO_2 is commonly used as an anesthetic in invertebrates.[15, 16] Many researchers utilizing invertebrates, particularly *Drosophila melanagaster*, sort genetic strains under a 100% CO_2 anesthesia. The cellular response to high CO_2 exposure or the resultant low pH may indeed have unintended consequences for physiological and behavioral studies. Exposing muscle or neurons to saline containing 100% bubbled CO_2 rapidly decreases pH_i. With the use of ion sensitive electrodes, it has been found that the pH_i in crab muscle and squid axons drops to around 6 and 5.7 respectively, when exposed to saline bubbled with CO_2.[17-19] The pH_o also decreases with CO_2 bubbling, thus effects on cellular function may be due to extracellular or intracellular changes in pH or directly by CO_2. Earlier studies indicate that molecular CO_2, not the pH_o or pH_i associated change, is in part responsible for alterations in synaptic receptivity at the neuromuscular junctions of crayfish and *Drosophila malanagaster*.[18, 19]

On the other hand, the action of protons (H^+) themselves on cellular function is well established from effects on ion channels and pumps to enzymatic cascades.[4] Protons can even increase the sensitivity of sensory neurons by increasing the opening of stretch activated ion channels (SACs), which are commonly used for mechanosensory transduction.[20] The fundamental SACs are TRP channels (Transient Receptor Potential channels), DEG/ENaCs channels (Degenerin/epithelial sodium channels, known to be present in invertebrates and vertebrates),[21] Piezo channels (pressure sensitive channels, found in plants and eukaryotic species)[22] and TMC channels (transmembrane channels, sound- and vibration-sensing hair cells in mice).[21] There are several reviews on SACs that provide more detailed descriptions of these channel types.[21-23]

Some SACs (i.e. DEG/ENaC channels) are altered by pH_o;[20] however, we have shown that the SACs in the crustacean preparations used in this study did not have enhanced excitability in response to low pH_o (5.0). To our knowledge no studies have yet addressed how pH_i will affect SACs in joint proprioceptors of the crustaceans, insects, or mammals. We approached this challenge by using known methodological procedures to alter pH_i by use of propionic acid as well as saline saturated with CO_2 gas.[5, 24, 25] Propionic acid as well as CO_2 will rapidly cross the bilipid membrane to acidify the cytoplasm; whereas altering the bathing saline pH to 5.0, with the use of HCl, does not result in pH_i becoming acidified in intact cells (see review).[4] Our past study documented that acute exposure (1 to 2 minutes) to pH_o of 5.0 only showed a slight reduction in activity depending on the type displacements used; however, after one hour exposure overall sensitivity to the displacements was significantly reduced.

The two model preparations we chose to use for this study are the mechanoreceptors associated with sensory endings embedded within chordotonal organs (COs), which monitor joint movements in the limbs of arthropods (insects and crustaceans). Specifically, we used the crab propodite-dactylopodite joint (PD) organ and the muscle receptor organ (MRO) in the crayfish abdomen in our analysis. Since these two preparations are well-described model systems for mechanoreception[26-32] we felt these preparations would be prudent to start to address the role of pH_i, pH_o and the effect of CO_2 on these joint proprioceptors.

The contribution of this study is to enhance the scientific understanding in the physiology of COs and the MRO in these crustacean preparations with regard to SACs and neuronal pH sensitivity by alterations in pH_i, pH_o and CO_2. The basic understandings may help to address the mechanistic underpinnings of mechanosensitive receptors in other organisms, such as muscle spindles in skeletal muscle of mammals and tactile as well as pressure (e.g., blood pressure) sensory receptors.

METHODS AND PROCEDURES

Crab

Blue crabs, *Callinectes sapidus*, were obtained from a local supermarket in Lexington, KY to which they were delivered from a distribution center in Atlanta, GA. They were bought and maintained in a sea water aquarium for several days prior to use in order to assess their health. All crabs used were alive and were very active upon autotomizing a leg for experimentation. While holding the crab with a net or large tongs across the carapace from behind, and avoiding the claws, a pinch across the merus of the walking leg with a pair of pliers would induce the leg to be autotomized. The leg was then placed in the Sylgard-lined dissecting dish and covered with crab saline at room temperature (21°C).

The chordotonal organ in the propodite-dactylopodite joint (PD) of the first or second walking legs of the crab was used. The details of the dissection and procedures are described in video and text.[33] After exposing the PD nerve and pulling the nerve into a suction electrode for recording the nerve activity, the dactyl was moved throughout the extended and flexed positions for several cycles with the aid of a wooden probe to ensure the nerve was not pulling on the chordotonal strand. A length of the nerve was left out of the suction electrode to provide slack.

The experimental conditions consisted of moving the dactyl from a flexed 90° angle from the propus to a full 0° in an extended (or open) position and then released. When the dactyl was released the joint would obtain a partial flexed position. Prior to the next displacement, the joint was flexed to the same starting position. The rates of movements were 0.5 sec, 1.0 sec, 2.0 sec and 4 sec for the 90° displacement with 5 sec between displacements. The analysis consisted of binning the responses into 0.5 sec periods for all the displacements and obtaining a count of spikes or a firing frequency of the nerve.

Crayfish

Crayfish (*Procambarus clarkii*), measuring 6–10 cm in body length, were used throughout this study (Atchafalaya Biological Supply Co., Raceland, LA). They were housed individually in indoor tanks. The details of the dissection and procedures are described in video and text.[34] The MRO nerve to either abdominal segment 2 or 3 was used in this study. The displacements used were from a relaxed position (similar to an extended abdomen in the intact animal) to a stretched position (similar to a flexed abdomen in the intact animal). The displacement rates were 0.5 sec and 4 sec for 5 mm distance. In addition, a stretch hold was used to obtain the static position sensitive response. The same electrode and signal recording technique was used as for the crab CO.

Saline and pharmacology

The salines used are the normal salines described previously.[33, 34] All bathing and experimental solutions were kept at the experimental room temperature of 21°C. Propionic acid was diluted from a stock and mixed in the saline appropriate for the species used. CO_2 was introduced by vigorously bubbling 100% CO_2 into the saline used for 10 minutes. The pH of the saline after bubbling was between 5.0 and 5.1. The saline was rapidly poured into the bathing dish and exchanged every 2 minutes with freshly CO_2 bubbled saline. All chemical compounds were obtained from Sigma (St. Louis, MO) and CO_2 was purchased from a local supplier (Scott Gross, Lexington, KY).

Electrophysiology

Suction electrodes made from glass pipettes fitted with plastic tips were used to record extracelluar signals from the cut nerves.[35] A P-15 amplifier (Grass Instruments) in conjunction with a PowerLab/4s A/D converter and Lab Chart 7 software (ADI Instruments, Colorado Springs, CO) obtained the signals to be recorded on a computer at a 10 or 20 kHz sampling rate. All data are expressed as a mean (± SEM).

To insure reproducibility in experimentation

The data collected in the classroom with students using 8 different physiological rigs was preliminary data in order to obtain an idea of what to expect for the different experimental conditions. The students made the recordings and analyzed the data. For standardizing the rate of the movements and analysis, all the data presented in the manuscript was obtained by 2 people (one conducting the movements and one marking the files on the computer. Every experiment had 6 trials with different preparations and was conducted over the summer of 2016 in a month period. One individual (V.D.) analyzed all the data sets so analysis would be consistent. The movements of the joints were performed by the same individual (R.C) for all trails. The movements were made by physically moving the joint and counting out loud: "one Miss" (0.5 sec), "one Mississippi" (1 sec), "two Mississippi" (2 sec), etc. We timed verbal counting on a stopwatch several times to ensure consistency in the speed of counting. Each time a movement was started or stopped, a mark on the file with a tap on the key pad would be recorded. To be sure the static holds were correctly measured, a set time of 7 seconds was analyzed as indicated by a time stamp on the acquisition software.

RESULTS

The two model proprioceptive organs used in this study are characteristic for a variety of types of proprioceptive structures. The PD joint in the crab walking leg contains the chordotonal organ referred to as the PD chordotonal organ (**Figure 1A**). The structure consists of an elastic strand that is attached to the proximal end of the dactylopodite on one end. The other contact point spans the joint attaching to the closer apodeme (invertebrates' tendon like structure in which skeletal muscle attaches). The neuronal sensory endings are embedded within the elastic strand to detect the movement of the strand. The MRO is arranged differently in that the sensory endings are embedded within muscle fibers that span the joint of the abdomen (**Figure 1B**). Within the sensory endings of the PD organ and the MRO are the SACs, which initiate ionic flux and depolarization of the neuron when they are deformed by the mechanical forces placed on them. The neurons within the PD organ and MRO respond differently depending on the rate and direction of movement as well as the static position of the joint. Schematic diagrams of the movements used in this study are shown along with the representative neural activity recorded from the whole nerve (**Figure 1**). The PD joint

was displaced from 90° to 0° at various rates (0.5 and 4.0 seconds). The same rates of movements were used for the MRO to provide a fast and a slow displacement. However, the anatomical arrangement is different so a direct correlation in firing rates of the neurons cannot be made between the two preparations. The general responses to the same environmental conditions can be compared. The displacement for the MRO was to a set position that mimicked flexion of the abdomen. Also, a static position of flexion (stretching of the MRO), which was held for 7 seconds, was used to index the neural activity and the effects of changing the extra or intracellular pH as well as the effects of exposure of CO_2.

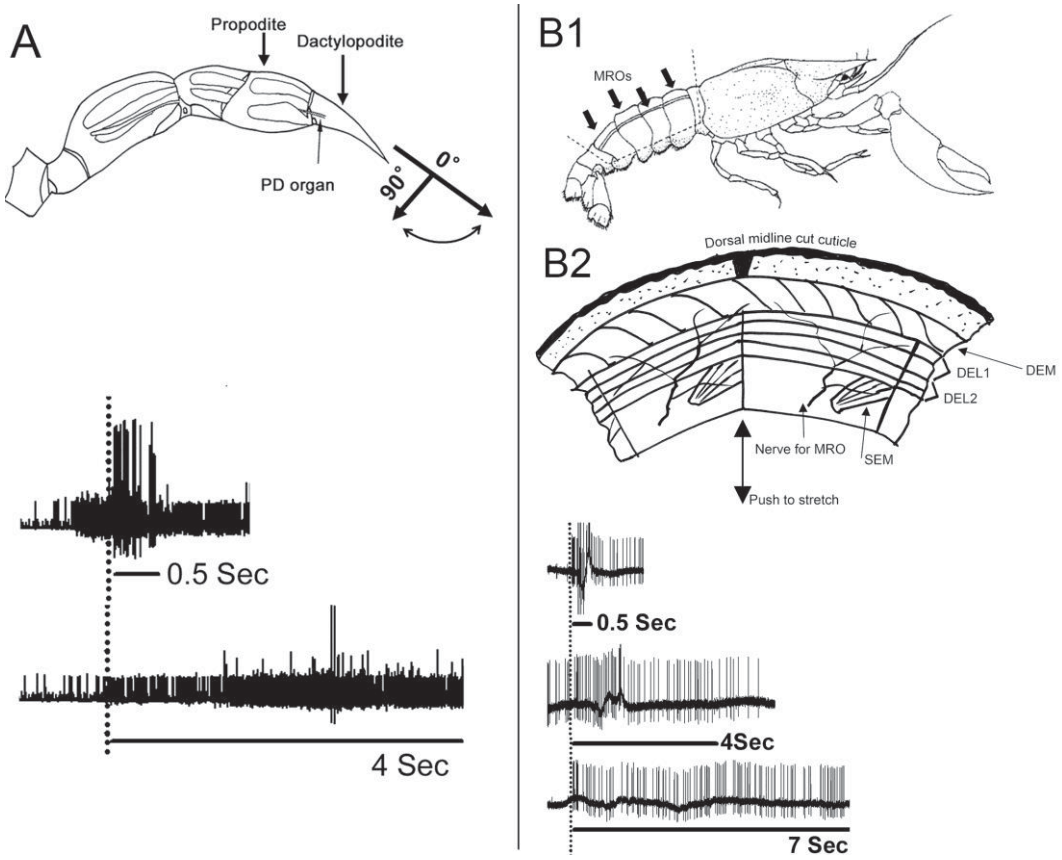

Figure 1. Anatomical arrangement of the displacements used for the PD organ of the crab walking leg (A) and the MRO of the crayfish abdomen (B). Either a stop pin or an anatomical position was used for consistency in the displacements. Rates of displacement for the crab joint were 0.5, 1, 2, and 4 seconds from 90° to fully extended (0°). B1: The MROs are located on the dorsal aspect of the abdomen. Movements for the MRO consisted of bending a joint in the hemi-longitudinal segment of the abdomen to a set location at a rate of 0.5 or 4 seconds as well as stretched and held for 7 seconds. B2: Two abdominal segments are illustrated. A schematic view of the deep extensor muscles (looking from ventral to dorsal) is provided. The particular muscles identified: deep extensor medial (DEM) muscles have a spiral fiber pattern, DEL1 is the first lateral group followed by the DEL2 muscles. The superficial extensor medial muscle (SEM) lies directly dorsal to DEL2. The two MRO muscles are more dorsal to the DEL1. The joint between the abdominal segments would be displaced at various rates to a set position while recording from the MRO nerve (the double arrow indicates where the joint between segments is located). Typical firing activity of the nerves is shown for a PD (top) and an MRO (bottom) preparation at each of the displacement rates. (Modified figure).[65, 66]

Low pH_o

Acute exposure to the bathing media at pH of 5 decreased the PD organ sensitivity for the 2 and 4 second displacements but after an hour at pH_o of 5, the sensitivity to the 1, 2 and 4 second displacements was also significantly reduced. The MRO did not decrease in responsiveness as much as the PD organ with the chronic exposure to low pH_o, but since there was such a wide variation in responses from preparation to preparation there was no consistent trend in the response to low pH_o. A representative plot showing the activity for saline, acute exposure to low pH_o, and chronic exposure (1 hr) is shown in **Figure 2A**. The trend in this representative preparation indicates that pH_o increased the spike activity; however, in comparing all 6 preparations there is substantial variability. Since each preparation was unique in the basal activity, a percent change within a preparation from saline exposure to low pH_o exposure was calculated for the acute and chronic exposure to low pH_o for all 6 preparations with the 3 displacements (**Figure 2B**). As indicated in **Figure 2B**, there was no consistent trend in increasing activity after acute exposure to low pH. In comparing the crab PD with the crayfish MRO, the percent change in the number of spikes for the chronic exposure to low pH_o is shown for the displacements, with the exception of showing the static hold for 7 seconds for the crayfish MRO since the static hold was not performed with the crab PD organ (**Figure 2C**).

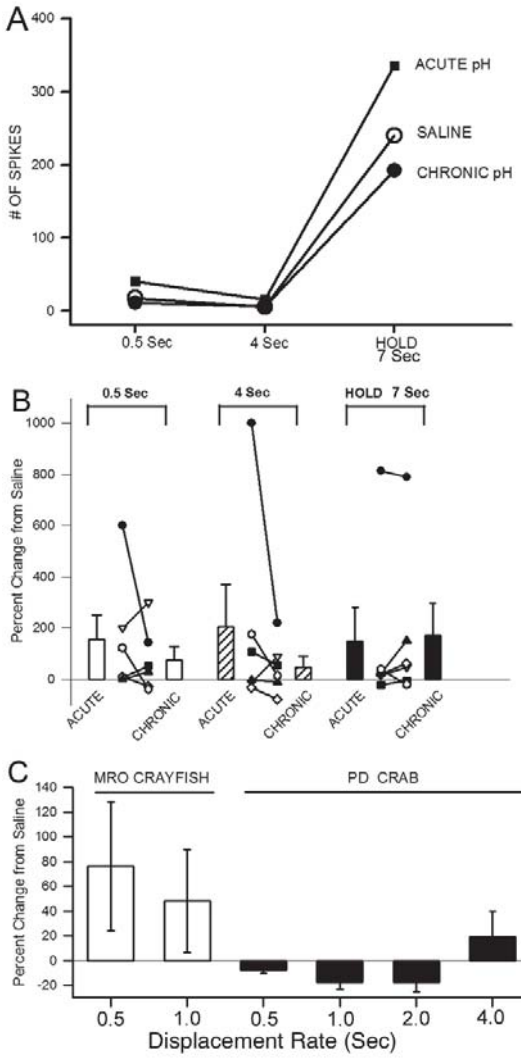

Figure 2. The effect of low pH_o on the sensitivity of joint proprioceptors. The rapid displacement within 0.5 second and 4 seconds did not demonstrate a large change in spiking differences for the MRO preparation (**A**). Static firing over a 7 second held position was also monitored for changes in spiking activity as shown for the MRO (**A**). A percent change from saline was used to compare the effects of low pH among preparations for the various displacement rates as shown for the MRO preparations (**B**). The same type of analysis was performed for the PD organ and the mean percent changes for both preparations are shown (**C**).

Propionic Acid

A common technique used to rapidly reduce pH_i is to expose preparations to saline containing propionic acid.[4, 5, 24, 25] This weak organic acid rapidly crosses the bilipid membrane and can also be exchanged back out of the cell by repeatedly changing the bathing media. A concentration of 20mM has been shown to produce a pH_i of about 5.61.[36, 37] We freshly added propionic acid to the saline prior to bathing the preparations. The same displacement rates were used for exchanging the pH_o. Representative spike activity from a crayfish MRO preparation and a crab PD organ is shown (**Figure 3** and **4**, respectively). The neural activity generally ceased within 5 minutes for the crayfish MRO preparations of all 3 displacements (0.5 sec **Figure 3A2**, 4 sec **Figure 3B2,** and the 7 sec hold **Figure 3C2**). The effect was also rapid for the crab PD but there was still lingering activity after 5 minutes for the same displacement rates (0.5 sec **Figure 4A2**, 4 sec **Figure 4B2,** and the 7 sec hold **Figure 4C2**). The range in neuronal responses, which displayed decreased activity throughout, implies that dynamic as well as static position sensitive neurons were affected. Both the crayfish and the crab preparations recovered well with exchanging the bathing saline a few times and repeating the displacement movements (see traces in **A3**, **B3** and **C3** in **Figures 3** and **4**).

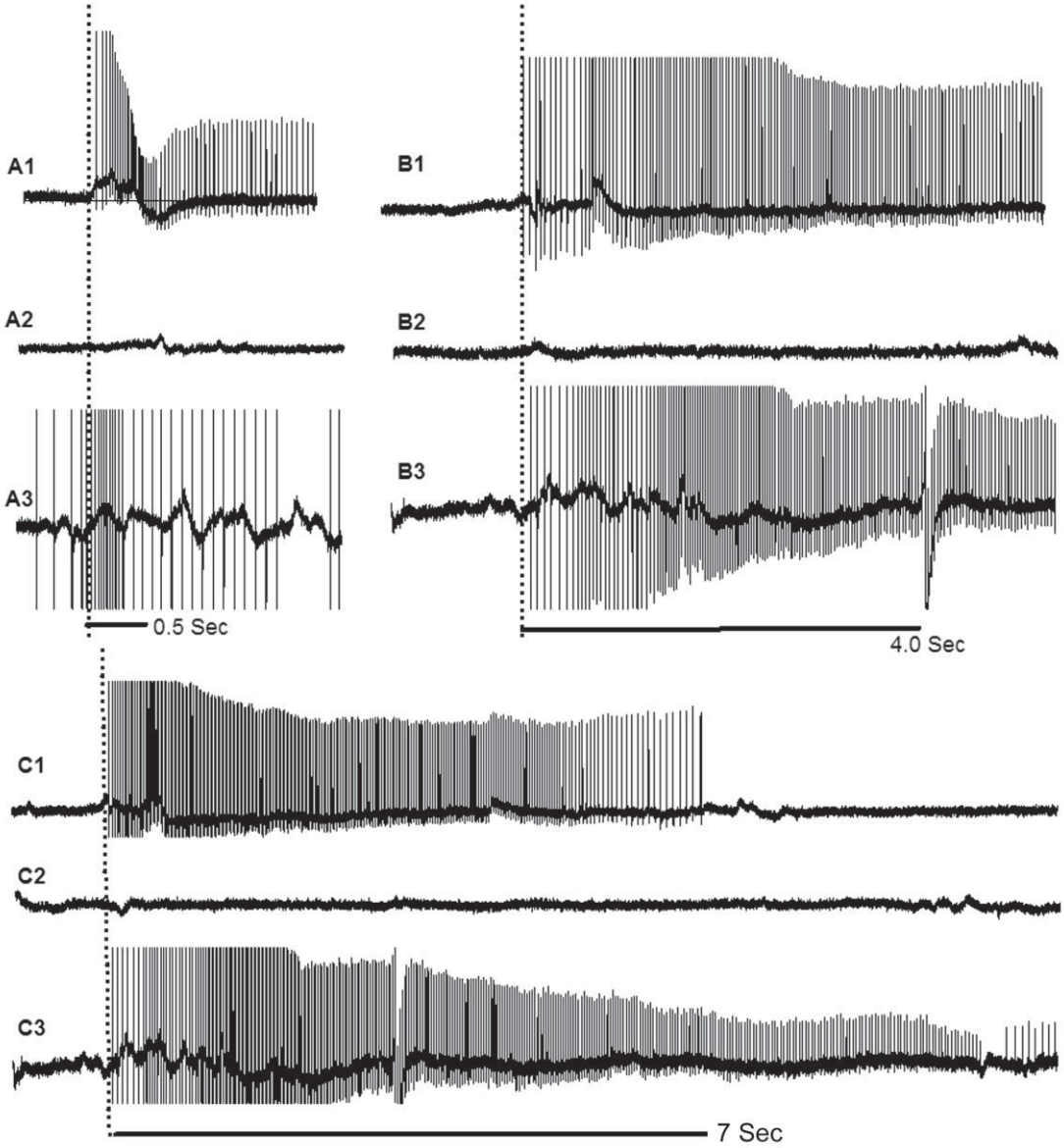

Figure 3. Representative traces in spiking for the different displacement rates and response to propionic acid exposure for the crayfish MRO. The 0.5 second displacement is shown in **A**, while the 4 second is shown in **B,** and the static held displacement of 7 seconds in shown in **C**. The responses in normal saline (**A1, B1, C1**) and during exposure to 20mM propionic acid (**A2, B2, C2**) as well as wash out with a return to normal saline (**A3, B3, C3**) are shown. The decrease in amplitude in **C3** is from the nerve being slightly displaced from recording electrode.

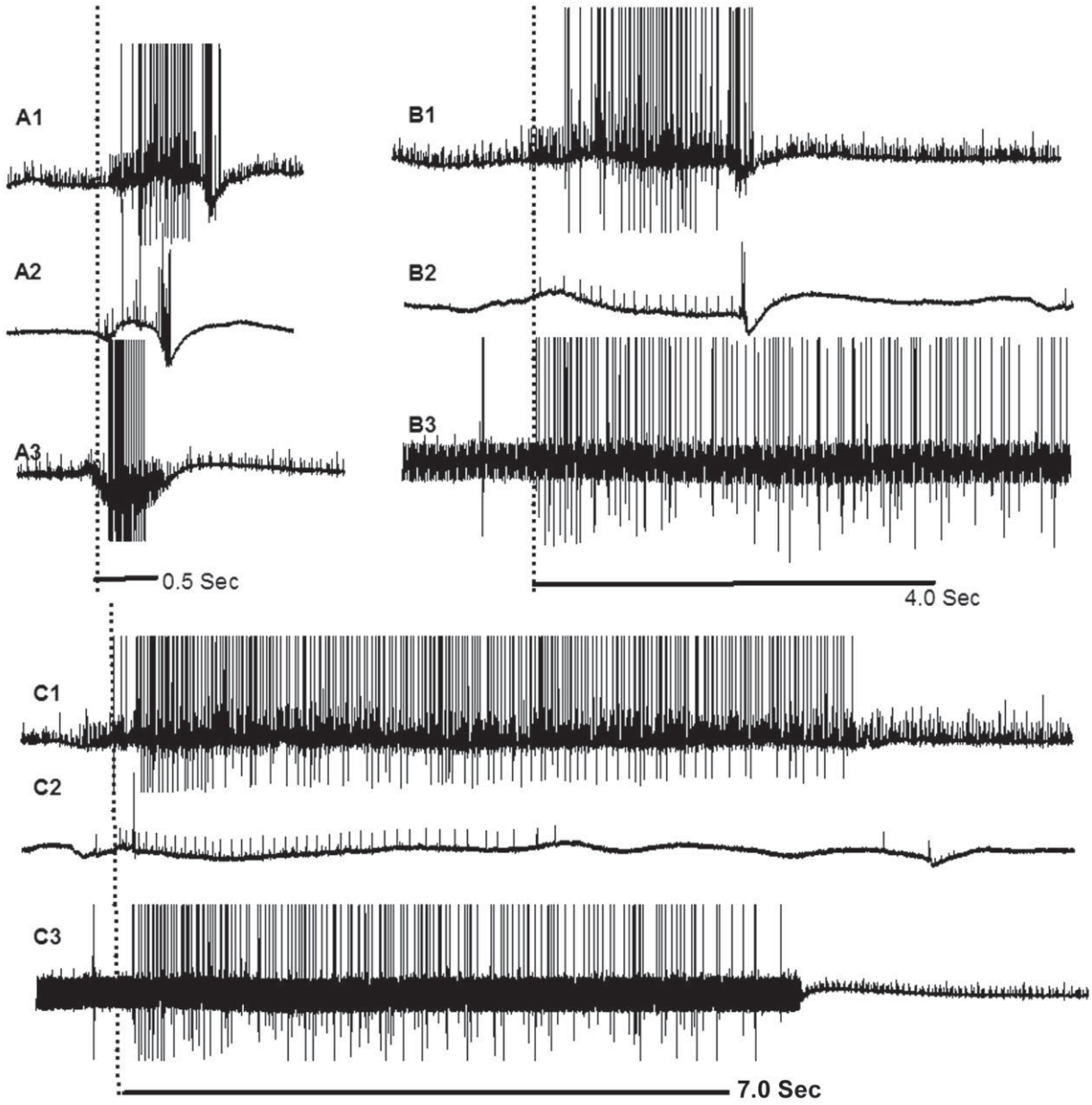

Figure 4. Representative traces in spiking for the different displacement rates and response to propionic acid exposure for the crab PD organ. The 0.5 second displacement is shown in **A**, while the 4 second is shown in **B**, and the static held displacement of 7 seconds in shown in **C**. The responses in normal saline (**A1, B1, C1**), during exposure to 20mM propionic acid (**A2, B2, C2**), and washed out with a return to normal saline (**A3, B3, C3**) are shown.

Since each preparation was unique in the basal activity, a percent change within a preparation to the saline exposure was calculated for the acute exposure to propionic acid for all 6 preparations with the 3 displacements for the crayfish MRO and crab PD organ. The trend in these preparations indicates that propionic acid reduces spike activity by nearly 100% in all 6 crayfish preparations and is greatly reduced in the crab PD for the 5-minute exposure (**Figure 5**).

Saline containing CO_2

As with propionic acid, the dissolved CO_2 in the saline rapidly crosses the bilipid membrane, perhaps quicker than propionic acid, where it acidifies the cell interior and reduces the pH in the saline bathing of the preparation. To maintain a high level of dissolved CO_2, freshly bubbled saline was exchanged often with the bathing media. The same displacement rates and procedures were used to examine the effects of exposure to CO_2 on the preparations.

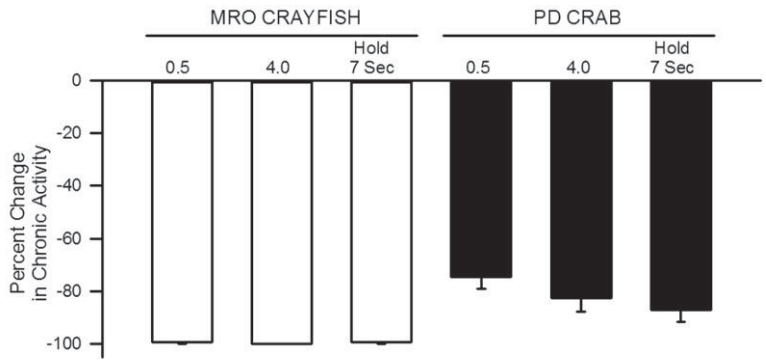

Figure 5. A percent change from saline was used to compare the MRO and PD preparations for the effects of propionic acid (20mM) for the various displacement rates and static held position.

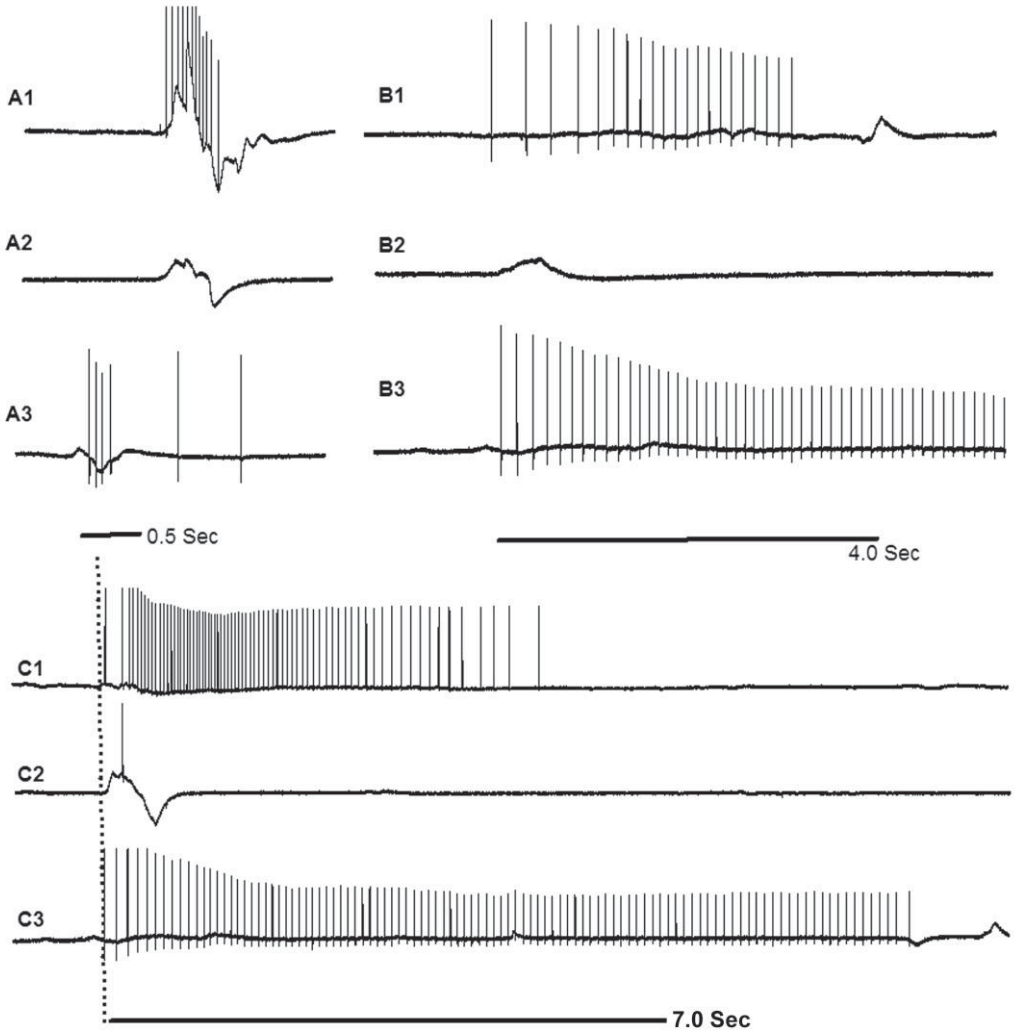

Figure 6. Representative traces in spiking for the different displacement rates and response to CO_2-containing saline exposure for the crayfish MRO. The half second displacement is shown in **A**, while the four second is shown in **B** and the static held displacement of 7 seconds in shown in **C**. The responses in normal saline (**A1, B1, C1**) and during exposure to CO2 (**A2, B2, C2**) are shown along with wash out steps with a return to normal saline (**A3, B3, C3**).

Representative MRO and a PD organ preparation are shown (**Figure 6 & 7** respectively). As with the exposure to propionic acid, the crayfish MRO preparations were more sensitive to CO_2 exposure than the crab preparation, displaying a more robust decrease in activity. However, it must be noted that only 2 sensory neurons are monitored in the crayfish as compared to approximately 80 neurons in the crab preparation. Just a few spikes could still be measured in 3 crayfish preparations with rapid displacements whereas the other preparations completely ceased in activity. The crab preparations were substantially affected as well but some activity could still be measured in all the preparations during the CO_2 exposure during one of the displacements rates (0.5 sec **Figure 7A2**, 4 sec **Figure 7B2**, and the 7 sec hold **Figure 7C2**) within the 5 minutes of exposure. Both the crayfish and the crab preparations recovered well upon saline exchange and repeated movements (see traces in **A3**, **B3**, and **C3** in **Figures 3** and **4**). The recovery from CO_2 was very rapid as compared to propionic acid exposure (i.e., recovery took place within approximately 1 minute after a single saline exchange vs. 3 or 4 exchanges of saline followed by a 5 to 10 minute waiting period after saline exchange from propionic acid in some cases).

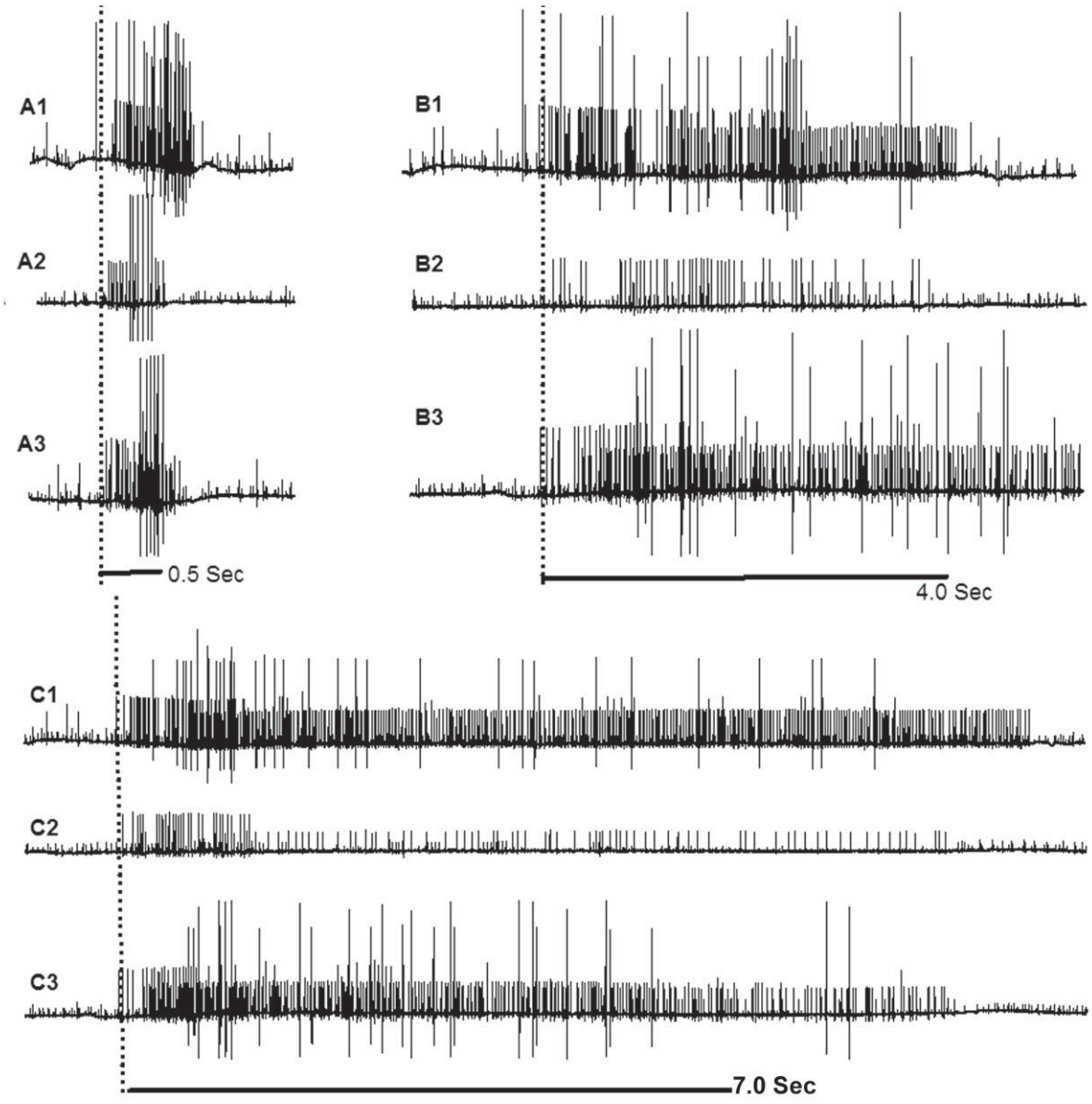

Figure 7. Representative traces in spiking for the different displacement rates and response to saline containing CO_2 for the crab PD organ. The 0.5 second displacement is shown in **A**, while the 4 second is shown in **B** and the static held displacement of 7 seconds in shown in **C**. The responses in normal saline (**A1, B1, C1**), during exposure to CO2 (**A2, B2, C2**), and washed out with a return to normal saline (**A3, B3, C3**) are shown.

As with exposure to low pH_o and propionic acid, a percent change within a preparation to the saline exposure was calculated for the acute exposure for all 6 preparations with the 3 displacements for the crayfish MRO as well as the for the crab PD organ (**Figure 8**). The trend in these preparations indicates that CO_2 decreased the spike activity in both sensory preparations (6 out 6 preparations). The overall percent reductions for the CO_2 exposures are not as large as for the propionic acid exposure (compare **Figures 5** and **8**).

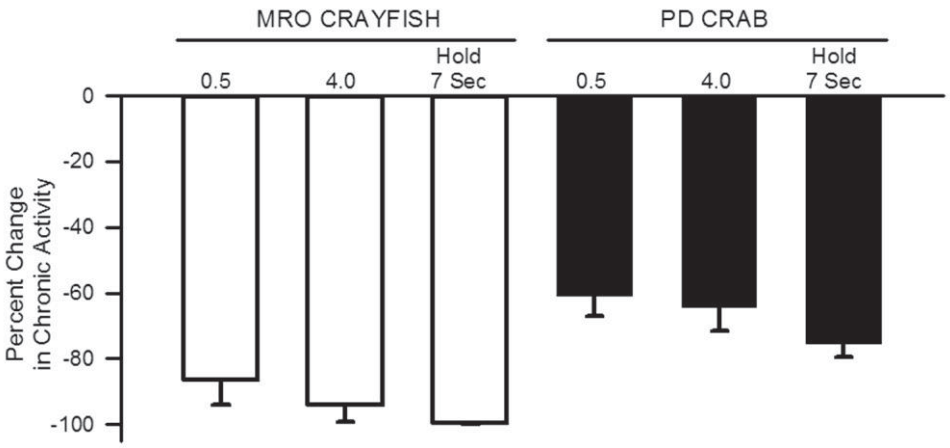

Figure 8. A percent change from saline was used to compare among the MRO and PD preparations for the effects of CO_2 exposure for the various displacement rates and static held position.

DISCUSSION

In this study we demonstrated the proprioceptive neurons associated with the crayfish MRO preparation and the PD organ of the walking leg in the crab are not highly sensitive to changes in extracellular pH, when pH_o is reduced to 5.0 from 7.4. After this reduction, the preparations maintained their firing rate over various velocities of movement and static positions, even though a slight increase in activity was observed after acute exposure. However, if intracellular pH is decreased by exposure to propionic acid or saline containing CO_2, there is a rapid decrease in firing rate in response to displacement. Recovery of activity is possible with re-exposure to normal physiological saline.

The exposure to high levels of CO_2 occurs commonly on insects and crustaceans used in research laboratories since it is an anesthetic. It is often used to anesthetize flies to sort them in genetic analyses. CO_2 induced pH-related neuronal depression could play a role in developing newer anesthesia modalities for arthropods. Therefore, it is paramount to enhance understanding of the detriments that may arise because of chronic exposure to CO_2. The chordotonal-proprioceptors within the joints of insects are analogous to the chordotonal-proprioceptors in the limbs of *Crustacea*. As stated earlier, the crayfish MRO is similar in structure and function to the muscle spindle proprioceptive organ in mammals, including humans. Since CO_2 is a by-product of cellular metabolism throughout the animal kingdom, understanding how exposure to CO_2 affects the two sensory model preparations used in this study can aid in addressing potential effects on neurons in other animals. Coupling energy metabolism and H^+ production within neural tissue was recently reviewed.[38]

The direct effects of CO_2 are difficult to study due to the rapid buffering of CO_2 and conversion to $HCO_3^- + H^+$.[3] In addition, the buffering abilities through proteins, organelle uptake and plasma membrane exchangers/pumps within neurons may vary.[4, 38] The solubility of CO_2 in solution varies with temperature and salinity so there are likely some differences in CO_2 within the hemolymph when exposing freshwater crustaceans and salt-water crustaceans to environments of a given CO_2 concentration. The higher osmolality saline used for the crab vs. the crayfish may also influence the amount of soluble CO_2; however, the effects observed in this study on the sensory neurons are similar for both animal models. The rapid decrease in neuronal activity upon CO_2 exposure, as with propionic acid, indicates a direct effect on either the SACs or in the ability of the neurons to produce action potentials or conduct electrical signals. Exposure to CO_2 did not significantly decrease the amplitude of action potentials in motor neurons of the crayfish, as measured with intracellular electrodes.[19] This suggests that the effects of CO_2 on the proprioceptors may be primarily on mechanosensory transduction or on the initiation of an action potential. It was noted that crayfish axons did not have an alteration in voltage gated Na^+ channel activation with changes in pH_o.[39] Thus, the induction of an action potential when past threshold should not be affected by pH_o but the effect of pH_i has not specifically been addressed in crab or crayfish neurons. However, it is known that depending on the state of activity of cells the effect of CO_2 exposure may have different effects depending on the membrane potential.[40, 41]

Since propionic acid and CO_2 exposure produced similar results of decreasing the sensitivity of the proprioceptors to the displacements and that both compounds can reduce pH_i rapidly,[4, 5] we postulate that low pH_i has an effect directly on the SACs or on the initiation of action potentials. Future investigations using direct injections of acid agents such as potassium acetate or HCl, which are cell impermeant, could address this possibility. The hardiness of invertebrate preparations to conduct such experiments is well known.[42-44]

Interactions between pH and other endogenous factors

Decreasing pH_o does not necessarily mean protons will diffuse into the cells and decrease pH_i since the bilipid membrane is not freely permeable to protons although protons can travel through various channels when they are opened. Cells can maintain a basic state even with an exposure to an acidic bathing medium and upon injection or acidifying cells with various approaches (e.g., injection of HCl, exposure to CO_2 and NH_4Cl pulses). They can readily return to a basic state following acidification of the surrounding environment. This indicates the strong nature of the cell to regulate proton balance through pumps and exchangers,[6] as demonstrated for crayfish neurons.[5] The low pH of 5 did not block the mechanical transduction of the stretch activated channels (SACs) in the sensory endings or the ability of the neurons to reach threshold and conduct action potentials. However, the fluidity of some bilipid membranes can be altered by small changes in pH_o.[45] The voltage gated Na^+ channel in the axon of crayfish appear to be insensitive to low pH_o within the range which would block acid sensitive SACs.[39] Additionally, we have shown in this study that these sensory endings and axons for the crayfish MRO and in the PD organ of the crab are not pH_o sensitive. The ability to maintain sensory responsiveness and electrical activity of these neurons demonstrates the robust nature of these crustacean preparations to an acute acidic extracellular or hemolymph environment.

There are neuro-active substances within the hemolymph of crustaceans that can alter the activity of sensory neurons. An earlier study demonstrated that serotonin and ecdysone, on their own as well as in combination, altered the activity of the neurons associated with the crayfish MRO.[46] The effects of serotonin and octopamine showed species differences in crustaceans in their effects on the MRO.[47] Peetz & Winter also reported on sustained activity in MROs when the hemolymph was mixed with a physiological saline, although no one particular substance was identified to be responsible for the action.[48] As for the chordotonal organs in crabs, if the hemolymph is mixed with a physiological saline and used as the bathing solution, the preparations last longer when isolated from the animal.[31] The species-specific saline without modulators or hemolymph present reduces unknown variables that may arise in an endogenous hemolymph. The effect of altered pH_o or pH_i, by CO_2 fluctuations, may vary within the animal due to buffering and the presence of neuromodulators within the hemolymph. In fact, we are not aware of physiological examination on the action of modulators over physiological measured ranges of pH known to occur within insects or crustaceans. In insects the pH of the hemolymph has been measured to be as low as 6.6 and for *Drosophila* the hemolymph (HL3) commonly used for physiology studies is pH 7.2 as was measured from isolated pooled hemolymph samples.[49, 50] However, it appears a physiological saline with a lower pH of 7.1 is substantially better for maintaining the heart function for exposed preparations.[51] In addition, heart rates increased in response to serotonin with a bathing saline that was buffered and maintained at 7.1.[51] In relation to mammals, we have not found any reports on the influence of pH_o and alteration in the effects of neuromodulation by serotonin or dopamine on neuronal function in humans. Receptors for neurotransmitters such as acetylcholine, GABA, and glutamate receptors do show pH sensitivity.[52] Several voltage gated ion channels also demonstrate pH sensitivity that is not assumed to be directly related to mechanical transduction.[53, 54] Such effects on the neural excitability and electrical conduction of axons related to the crayfish MRO or proprioceptors in the crab have not been investigated. As we report, the SACs in the sensory endings do not appear to show a change in mechanosensory transduction with pH as low as 5.0 as measured by the frequency of the extracellular recorded spikes. The amplitude and shape of the action potentials may indeed be altered but we were not able to detect consistent differences by monitoring the spikes. However, the effect of pH_o on neuronal activity depends on the type of neuron as some are known to be very sensitive to pH_o changes which may occur due to indirect effects through influences on ion channels.[55, 56]

Since lowering pH_o does not directly address the effects of a lower pH_i, investigators have used various means to induce reductions in intracellular pH to address the physiological effects. Injecting substances: cell permeant compounds such as potassium acetate, propionic acid, or exposure to CO_2, have all been utilized to reduce pH_i.[4, 5, 24, 25, 57] Exposure to propionic acid as well as the bathing fluid containing CO_2 rapidly decreases pH_i from a basal state and the effect is rapidly reversed in removing these compounds.[4, 5] Since these compounds reduce pH_i in addition to pH_o, any physiological alterations might be due to a combined effect, rather than a sole reduction in pH_i. The acute effect of propionic acid in our case was rapid and since the frequency of spikes drastically decreased we did not pursue the effects of repeated exposure times. Constant exposure for an hour, in propionic acid at the concentrations used, did not allow the neurons to recover. Long exposures to propionic acid may indeed cause effects that cannot be so readily reversed due to the ability of propionic acid to also transverse cellular organelles, such as mitochondria, within the cell.

Comparative differences in the proprioceptors

Differences in the anatomical arrangement of the crayfish MRO and the crab PD can complicate direct comparisons with addressing effects on mechanosensory transduction since the sensory endings of the MRO are embedded within muscle. Thus, any effects on the excitatory or inhibitory motor neurons that innervate the muscles can alter the forces exerted on the sensory endings. However, the conditions addressed in this study with pH_o, pH_i and CO_2 showed similar outcomes for both preparations. More refined recordings in the graded responses within the sensory endings could be different for the MRO preparation exposed to low pH_i or pH_o, by HCl adjusted saline or propionic acid, as compared to saline containing CO_2. CO_2 exposure was shown to block glutamate receptors at neuromuscular junctions whereas spontaneous events and evoked events are still present at neuromuscular junctions with low pH induced by other means.[18, 19] Thus, any decrease in baseline response in muscle tension from a lack of responding to the spontaneously released glutamate might reduce the basal tension on the sensory endings. Low pH_o does produce some depolarization in muscle for insects and crustaceans,[18, 19] and thus could produce an increase in force production if the motor nerve was stimulated to produce muscle contraction. The conditions used in the current study were created by passively moving the joints and were not dependent on motor nerve stimulation, although any enhancement of the terminal depolarization, even in the transected motor axons, could have an impact on muscle contraction and tension on the sensory endings for the MRO preparation. These confounding factors do not arise in the crab PD preparation, as the sensory endings are readily exposed in the elastic strand connecting the propodite and dactylopodite. Additionally, the crayfish MRO within a hemi-segment is only comprised of two sensory neurons while the crab PD is comprised of around 80 different neurons. The sensory somata as well as the axons are much larger in the MRO preparations as compared to the PD neurons for the animals used in these studies. Buffering abilities within neurons of various sizes may be different and likely effects on input resistance of the cells are varied due to size differences. The depolarizations required to reach threshold for generating action potentials are likely different in the neurons of varied size.

Potential translational implications

In considering the possible translational implications of the findings in this study to those of mammals, one can readily relate them to neuronal pathophysiology in ischemia, hypoxia, and lowered pH_o and pH_i due to CO_2 imbalances.[58] An active area of research is elucidating the mechanisms behind a ketogenic diet decreasing the occurrences of epilepsy,[59-61] which is known to be influenced by pH.[62]

CONCLUSIONS

This analysis investigates potential mechanistic explanations for spreading depolarization and synaptic depression when tissue is not perfused well to relieve the altered pH, ionic spillage, and CO_2 accumulation which occurs from damaged cells or compartmentation due to swelling and reduced vascular perfusion outside the initial tramatome.[63] Recent studies in addressing effects of neurons outside the site of initial injury focus on possible mechanisms, such as K^+ and other ion/intracellular component spillage, which would leak from damaged cells and could alter healthy cell excitability.[64] However, little attention is given to the possible detriments of CO_2 accumulation around surrounding cells. A synergistic effect may arise in situations in which injured cells dump intracellular ion and amino acid stores, leading to increased cellular activity through depolarization of healthy cell membranes, which could then enhance CO_2 production and exacerbate cellular damage. As for muscle spindles associated with proprioception in mammals, a similar situation can arise. Compartmentalized muscle following injury lacks proper vascular perfusion. Thus, CO_2 buildup may assist in damage, not only to the muscle fibers, but also to the sensory neurons positioned within the muscle spindles. Similar outcomes could theoretically arise in neural tissue following traumatic brain injury and/or in COPD patients who experience systemic alteration in pH. It may be beneficial to conduct future studies which measure muscle spindle activity in muscles with damaged extrafusal fibers or in *in situ* preparations where solution pH can be altered experimentally.

ACKNOWLEDGMENTS

This work was funded by student laboratory fees for a course taught in Department of Biology, University of Kentucky and personal funds (RLC).

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ABOUT THE STUDENT AUTHORS

Many of the authors were students in a neurophysiology lab-based class addressing authentic scientific based questions in regard to the topic of examining how extracellular and intracellular pH would influence proprioception. Most of the undergraduate students have now graduated with a BS in biology or neuroscience.

PRESS SUMMARY

Two invertebrate model preparations are used to demonstrate how external and internal cellular pH changes as well as exposure to CO_2 can alter neuronal function for proprioception. Proprioceptive neurons are most affected when intracellular pH is decreased by exposure to propionic acid or saline containing CO_2. The ease in recording from these neurons can help researchers understanding mechanistic properties of mechanosensitive receptors in other organisms, such as muscle spindles in skeletal muscles of mammals and tactical as well as pressure (i.e., blood pressure) sensory receptors.