Stretch activated channels in proprioceptive organs of crab and crayfish are sensitive to gadolinium but not amiloride, ruthenium red or low pH

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The type of stretch activated receptors (SARs) in the chordotonal organs in the crab walking leg and of the muscle receptor organ (MRO) in the crayfish abdomen have not yet been classified as to their molecular or pharmacological profile. The purpose of this study is to examine the pharmacological profile of SARs in the proprioceptive neurons in the crab and crayfish models. Since many SARs share the pharmacological profile of displaying low pH or being proton sensitive (i.e. being more active) or blocked by the diuretic amiloride or ruthenium red as well as being blocked by the broad stretch activated channel blocker gadolinium (Gd^{3+}), we used these agents to screen the receptors. Various displacement rates as well as static positions that activate the stretch activated receptors were used in examining their pharmacological profiles. Hour-long exposure to low pH decreased neural activity of the chordotonal organ of the crab more so than to amiloride or ruthenium red. The crayfish MRO did not show pH sensitivity or sensitivity to amiloride or ruthenium red. Gd^{3+} rapidly blocked neural activity in both the crab and crayfish. It appears these stretch activated receptors may not have a classification that is suited to the standard pharmacological profiles. The molecular makeup of the channels also awaits characterization. This could reveal a novel SAR subtype. Our neurophysiology course[1] took this project on as a course-based undergraduate research experience (CURE) to address an authentic research question.

Abbreviations: ASC-acid sensitive stretch activated channels; CO-chordotonal organs; MRO-muscle receptor organ; SACs-stretch activated ion channels; SARs-stretch activated receptors; sec-second.

Keywords: Proprioception, sensory, invertebrate, pharmacology

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Introduction

Proprioceptors are a group of specialized receptors that detect position and movement (kinesthetic). They monitor joint position, direction, speed, and muscle length. Arthropods, like vertebrates, have articulated appendages. It is therefore, not surprising that the proprioceptors described for vertebrates have their counterparts in arthropod limbs and joints.

The physiology of mechanosensory transduction is diverse since there are many types of receptors that transduce mechanical forces into electrical neural impulses in all organisms with a nervous system.

Receptors that are sensitive to mechanosensation are used to monitor both external and internal forces and are essential in transferring information that allows for appropriate behavioral responses to external stimuli and body positioning. Invertebrates serve as models in understanding the physiology of mechanoreception due to the diversity of receptor types and the relative ease with which one can utilize these models in experimentation. The family of stretch activated ion channels (SACs) is broad and currently being characterized by gene/protein sequences and pharmacological profiles (Boscardin et al., 2016).

The SACs, which are mechanoreceptors, are associated with sensory endings embedded within chordotonal organs (COs). The COs monitor joint movements in the limbs of arthropods (insects and crustaceans). The muscle receptor organ (MRO) in the crayfish abdomen is a well-described model system but only preliminary studies have been conducted examining the pharmacology of these receptors. In addition, the pharmacological profiling of the SACs within COs in the limbs of crabs has not previously been investigated.

The biological field is still tackling a classification scheme for various forms of SACs. Pharmacological profiling is aiding in dividing the subtypes; however, there are still unique profiles being uncovered that do not follow a strict pattern. There are several reviews dedicated to classification and characterization of SACs (Ernstrom and Chalfie, 2002; Arnadottir and Chalfie, 2010; Coste et al., 2010; Geffeney and Goodman, 2012). The fundamental SACs are TRP channels (Transient Receptor Potential channels), DEG/ENaCs (Degenerin /epithelial sodium channels; known to be present in invertebrates and vertebrates; Geffeney and Goodman, 2012), Piezo (pressure sensitive channel; found in plants and eukaryotic species; Coste et al., 2010) and TMC (transmembrane channels; sound- and vibration-sensing hair cells in mice; Geffeney and Goodman, 2012).

The crab CO is ideal for pharmacological studies of mechano-transduction since sensory endings are embedded in an elastic strand with cell bodies and the endings relatively exposed to hemolymph (Hartman and Boettiger, 1967; Alexandrowicz, 1972). The sensory endings, which transduce the mechanical forces through SACs, are arranged in a very typical pattern within the elastic chordotonal strand in various species of crab. The orderly arrangement of the endings, with defined spacing and placements within the elastic strand allows them to provide a range fractionation (i.e., sensitive to set displacement angels of the joint) for static position and dynamic displacements (Whitear, 1962, 1965; Hartman and Boettiger, 1967; Hartman and Cooper, 1994; Cooper and Hartman, 1999; Cooper, 2008). The propodite-dactylopodite joint (PD) offers a readily accessible CO and ease in monitoring joint displacement while recording from the associated CO nerve. The COs are named according to which joint they are monitoring (i.e., "PD-organ" is the organ between the propodite (P) and the dactylopodite (D)) of the leg (Whitear, 1960; Alexandrowicz, 1967).

The crayfish MRO is more complex as the sensory endings are embedded within muscle fibers. When the muscle fibers are stretched, the displacement stretches the sensory ending and opens SACs. The anatomical arrangement and physiology is in some regards very similar to the muscle spindles in mammalian skeletal muscle (Kuffler, 1954). In mammals the sensory endings associated with the intrafusal spindles show an ionic behavior similar to the DEG/ENaC channel subtype (Bewick and Banks, 2015). There are two types of

sensory neurons, each associated to their own distinct muscle fiber. One MRO is referred to as the rapidly adapting neuron (the neuron stops firing quickly even while the stimulus is still present) and the other as the slow adapting neuron (slowly decreases the firing with a given maintained stimulus) (see Rydqvist et al., 2007 for a review).

We examined the effect of common pharmacological agents, known to block or activate SACs in crustacean preparations, in order to better characterize their stretch activated channels. Since these invertebrate preparations are also commonly used as models for neurophysiology teaching (Leksrisawat et al., 2010; Majeed et al., 2013), the identification of the type of mechanosensory transduction is beneficial for educators using them in teaching pharmacology and physiological principles. Amiloride and ruthenium red are two commonly used agents to block a subset of SACs (Coste et al., 2012; Omerbašić et al., 2015). Some receptor subsets of the TRP family, including those likely to be in the proprioceptive organs of crustaceans used in this study, are known to be blocked by ruthenium red, while the DEG/ENaC channels are blocked by amiloride (although some in this subset are only blocked by high concentrations of amiloride (Omerbašić et al., 2015)). In addition, low extracellular pH (5.0) is known to activate some forms of the DEG/ENaC channels referred to as acid sensitive stretch activated channels (ASC) (Welsh et al., 2002). In addition, some ASC are also blocked by amiloride (Omerbašić et al., 2015). The classified Piezo channels are comprised of a distinct type of protein sequence and are similar among species from mammals to invertebrates. They are now considered a novel class of ion channels involved in mechanotransduction through stretch activation (Coste et al., 2012). However, the mammalian form is blocked by ruthenium red while the form in *Drosophila* is not (Coste et al., 2012). Although, the Piezo associated channels in dorsal bipolar dendritic neurons, which monitor the stress on the cuticle of the larval *Drosophila*, and likely serve some role in proprioception as well are blocked by amiloride (Suslak et al., 2015). Gadolinium (Gd^{3+}), a non-selective blocker for SACs, is known to block a variety of SACs, some of which are unique in pharmacology. One such example is the baroreceptor neurons in mammals (Halduezok et al., 1994). Thus, Gd^{3+} was also used in this study to assay the effect on these proprioceptors. The SACs associated with crustacean COs and MRO may be unique in structure and pharmacological identification.

Our goal is to enhance understanding of the physiology of COs and the MRO in these crustacean preparations, which serve as models for mechanosensory transduction. It is likely that the SACs function similarly and show similar pharmacology throughout crustaceans and insects.

Material and Methods

Crab

Blue crabs, *Callinectes sapidus*, were obtained from a local supermarket in Lexington, KY, USA which were delivered from a distribution center in Atlanta, Georgia, USA. They were bought and maintained in a seawater 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 induced the leg to be autotomized. The leg was then placed in a Sylgard-lined dissecting dish and covered with crab saline at room temperature (21^{o}C).

The CO 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 by Majeed et al. (2013). 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 (Figure 1A).

The experimental conditions consisted of moving the dactyl from a flexed 90 degree angle from the propus to a full 0 degrees in an extended position (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 with 5 sec between displacements. The analysis consisted of binning (i.e., counting all responses in groups) the responses into 0.5 sec periods for all the displacements and obtaining an overall count of spikes. This allows for a firing frequency to be calculated. The movements were made by use of a wooden dowel placed against the end of the dactyl for displacing the PD joint while extending the leg. When one moved the joint one would count out loud: one- Miss (0.5 sec), one-Mississippi (1 s), two- Mississippi (2^{nd} s), etc… The displacements were marked on the computer file recording the neural signals.

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 (Leksrisawat et al., 2010). The MRO nerve to either abdominal segment 2 or 3 is used in this study. The displacements used are 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) (Figure 1B). The movements were made by use of a wooden dowel to push on the segmental cuticle to stretch the MRO. An insect dissecting pin was placed in a Sylgard-lined dish as a stop mark for the range of displacement. When one moved the joint one would count out loud: one- Miss (0.5 sec), one-Mississippi (1 s), two-Mississippi (2^{nd} s), etc… The displacements were marked on the computer file recording the neural signals. Since the arrangement of the abdominal joints is different than the crab limb only 0.5 sec and the slow 4 sec displacements was used. In addition, a static hold while bending the joint was used since there was usually low activity with the half and 4 sec displacements. 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 (Majeed et al., 2013; Leksrisawat et al., 2010). Crayfish saline: a modified Van Harreveld's solution (in mM: 205 NaCl, 5.3 KCl, 13.5 $CaCl_2 \cdot 2H_2O$, 2.45 $MgCl_2 \cdot 6H_2O$, and 5 HEPES adjusted to pH 7.4). Crab saline: solution (in mM: 470 NaCl, 7.9 KCl, 15.0 $CaCl_2 \cdot 2H_2O$, 6.98 $MgCl_2 \cdot 6H_2O$, 11.0 dextrose, 5 HEPES acid and 5 HEPES base adjusted to pH 7.4). All bathing and experimental solutions were kept at the experimental room temperature of 21°C. Amiloride and ruthenium red were used at a concentration of 1mM. This concentration was used since it has been shown in earlier studies to be effective in blocking SACs

(Delmas et al., 2011). The pH of the saline was reduced to 5.0 with HCl for testing the effect of low extracellular pH (7.5 and 7.4 are normal pHs for crab and crayfish, respectively). Gadolinium chloride ($GdCl_3$) was used at 100 μM and 1 mM to determine if there was there is a dose dependent effect. All chemical compounds were obtained from Sigma (St. Louis, MO, USA).

Electrophysiology

Suction electrodes made from glass pipettes fitted with plastic tips were used to record extracelluar signals from the cut nerves (details of making the suction electrodes is provided in Baierlein et al., 2011). 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, USA) obtained the signals to be recorded on a computer at a 10 or 20 kHz sampling rate. The neural activity is readily determined from noise since when the CO or MRO is relaxed and held still, there is no neural activity.

Statistical analysis

All data are expressed as an average value along with the standard error of the mean (i.e., $\pm$ SEM). The rank sum pairwise test was used to compare the differences in neural activity before and after exchanging a solution with saline containing the compounds or lowered pH. This analysis was performed with Sigma Stat software. P of $\leq$ 0.05 is considered as statistically significant.

Results

The arrangement of the crab walking leg allows one to easily move the joint to set positions in terms of a displacement angle. When fully extended the dorsal alignment is linear (0 degree bend at the joint), and when flexed, a 90^o angle is obtained as schematically shown in Figure 1A. The proprioceptors for the PD organ are stretched when the joint is extended. The electrical activity of the PD nerve, recorded with an extracellular suction electrode, is comprised of various amplitude spikes. The amplitudes are related to the axonal sizes (Hartman and Boettiger, 1967) and there can be 50 to 100 neurons within a PD organ depending of the age of the crab (Hartman and Cooper, 1994). For quantification of the neural activity, a threshold above the baseline is used, which measures all neuronal activity throughout the displacements. Some of the neurons in the crab chordotonal organs only fire during the initial displacement and are velocity sensitive (i.e., dynamic sensitive neurons), while the neurons with smaller somas and axons, which give rise to smaller spikes, monitor the static positions. Thus, some of these static position sensitive neurons are recruited at different displacement positions and can maintain their firing at a given position with little accommodation (Hartman and Boettiger, 1967; Cooper and Hartman, 1999). The various displacements we use and representative spike activity is shown in Figure 1A.

It is not as easy to note the displacement angle in the MRO in the crayfish abdomen. To maintain consistent degree of displacement the abdomen is cut along the dorsal midline and the longitudinal half is pinned in such a manner that 4 hemi segments have a slight bend but also allow the middle to be displaced by pushing on the cut cuticle on the ventral side. The dorsal cut is stretched until a marker pin is contacted. The middle segment moves from the two adjacent segments in a triangle-like shape with the wider angular movement on the dorsal side. This results in the MRO being elongated while recording from the MRO

nerve. The muscle strands of the MRO are more dorsal to the DEL1 muscle. Thus, underneath the DEL1 as shown in the ventral view depicted in Figure 1B2. The two neurons give rise to two different amplitude spikes in most recordings and the rapidly adapting neuron stops firing sooner than the slowly adapting one with a constant stretch of the strands. In assessing the neural activity, the number of spikes from both neurons is counted during the displacements. Since it is more difficult to move the abdominal segments with as much precision as the PD joint, only a rapid 0.5 sec movement and a slow 4 sec movement is used in addition to stretching the joint and holding it for 10 sec. The neural activity is measured by setting a threshold above the baseline to count the number of spikes from both neurons.

Crab PD Organ

The spike activity from the PD nerve is complex since there are various amplitudes of the events due to the large number of neurons within the PD nerve (Figure 1B1). Representative responses with the various treatments are shown for the 1 sec and 4 sec displacements. The displacement is from the bent position to the elongated (0 degrees). In each condition (pH 5.0, amiloride 1mM, and ruthenium red 1mM) the activity is analyzed for each of the displacement rates (0.5, 1, 2 and 4 sec). Representative traces for the effect of low pH of 5.0 are shown (Figure 2). The low pH did not cause the activity to increase even after 1 hour of being exposed. Thus, the SACs are not increased by low pH (5.0). The type of analysis for a representative preparation shows the number of spikes counted within each 0.5 sec period (Figure 3). This is shown for the saline exposure prior to changing to a saline with low pH and for the activity after 1-hour exposure to the low pH. In some preparations the activity is even more prominent with exposure to low pH but on average there is no consistent change for the 0.5 and 1 sec displacements. However, it did appear that the 4 sec displacement might be showing a greater decrease in activity over time. To investigate this more carefully, the spike activity within time periods of 0.5 sec bins was measured for each displacement rate and for each compound tested. Notice the activity for the slow 4 sec displacement rate in the low pH saline after an hour did not vary much from the initial activity when bathed in saline or the acute pH exposure (Figure 2). However, further along in the displacement (closer to being fully extending of the joint) there is enhancement. In examining the activity profile for the 0.5 sec bins for each preparation there is no consistent trend in the activity profile over time. Overall, the activity appeared to be decreasing in spite of some 0.5 sec bins showing increased activity for the chronic exposure to low pH. Thus, the total number of spikes, which occurred for the entire displacement times, is measured and compared. Since each preparation has a different number of spikes for the displacements in saline, a percent change from the initial saline exposure to low pH for the acute and chronic exposures is used for comparisons (Figure 4). The percent changes for each individual preparation is shown as symbols on the graph along with the average changes for the 6 preparations. There are no significant differences by comparing the general distributions due to the wide variation in the responses for each displacement. However, 6 of 6 preparations showed an overall decrease in the spike activity for the 2 and 4 sec displacements with the acute low pH exposure. Also, 6

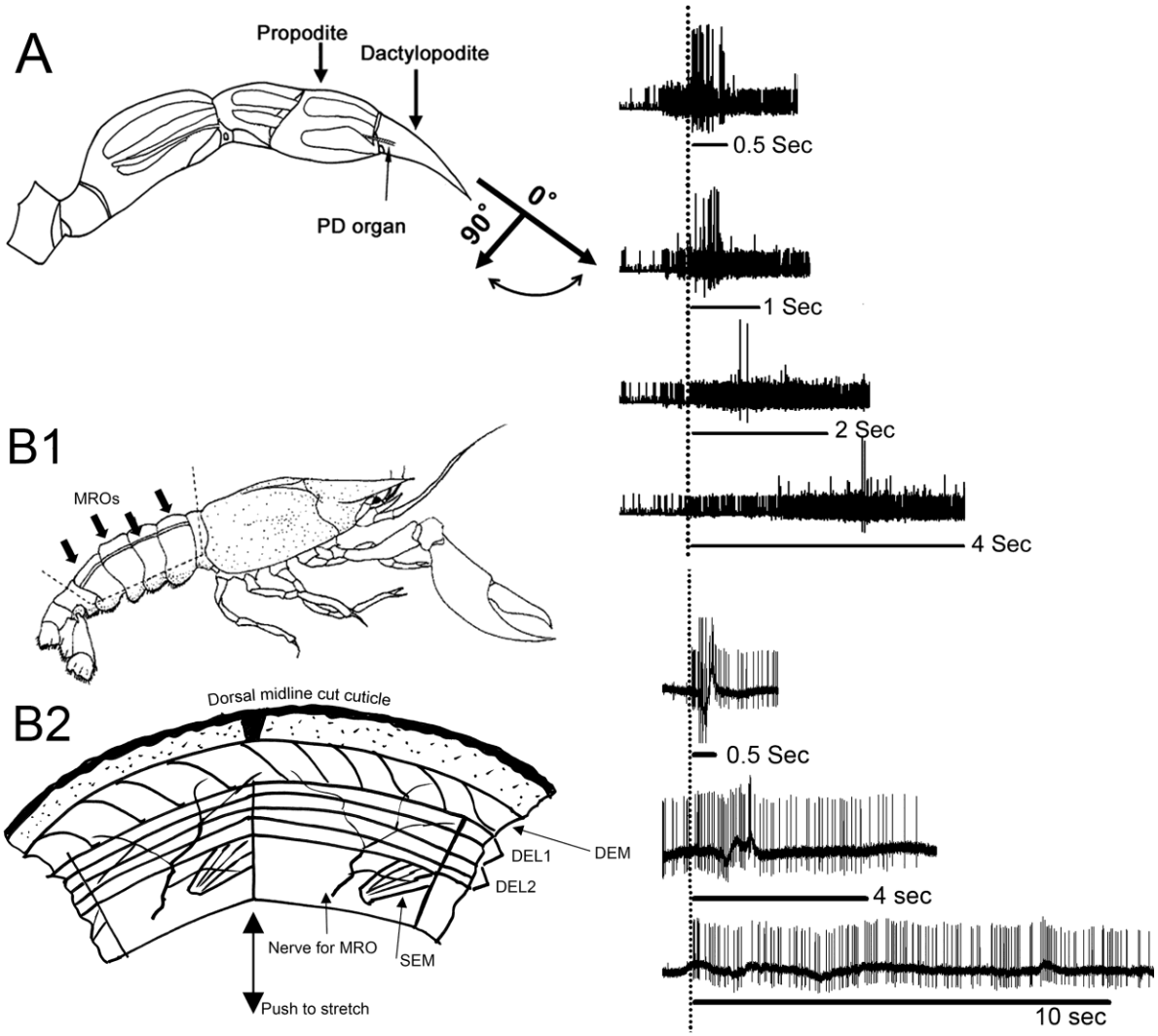
A
Propodite
Dactylopodite
PD organ
90°
0°
0.5 Sec
1 Sec
2 Sec
4 Sec
B1
MROs
B2
Dorsal midline cut cuticle
DEM
DEL1
DEL2
Nerve for MRO
SEM
Push to stretch
0.5 Sec
4 sec
10 sec

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 sec from 90^0 to fully extended (0^o). (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 abdomen to a set location at a rate of 0.5 or 4 sec as well as stretched and held for 10 sec. (B2) two abdominal segments are illustrated. A schematic view of the deep extensor muscles (looking from a ventral to dorsal). 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 (shown with the double arrow is where the joint between segments is located). Typical firing activity as observed in all the preparations of the nerves is shown for a PD (top) and a MRO (bottom) preparation at each of the displacement rates. The dotted vertical line represents the start time of the displacements.

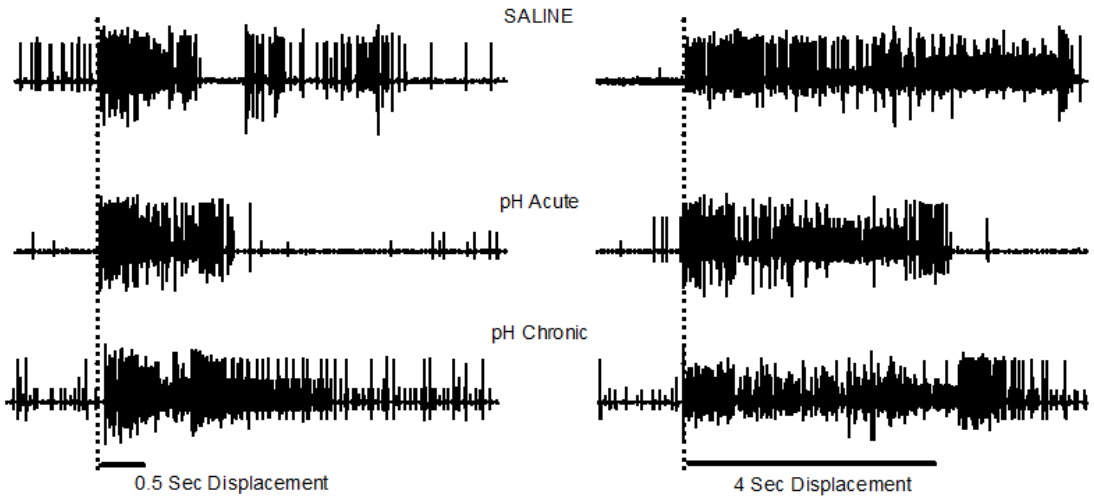

Figure 2: Representative recordings obtained from the crab PD nerve with exposure to low pH saline. The nerve activity for the 0.5 and 4 sec displacements are shown. The acute response after changing the bathing media and response after 1 hour exposure (chronic) to low pH (5.0) are shown.

of 6 preparations decreased activity for all displacements after chronic low pH exposure. Since some of the preparations show very little decrease we are cautious to imply that the SACs are low pH sensitive. The results indicate that the SAC's are not increased nor are the channels blocked by low pH since there is still a pronounced effect of movement even when the responses are decreased in activity over time with low pH exposure.

In comparing the effects of the two other chemical agents on the activity of the SACs the same analyses is performed for acute and chronic exposures.

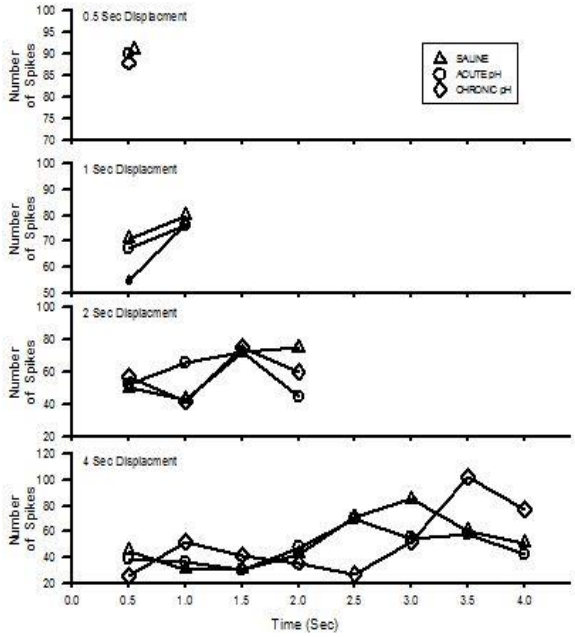

Figure 3: Analysis of the activity for the various displacement rates for a representative PD preparation of the crab leg. The spike activity within time periods of 0.5 sec bins was measured for each displacement rate. The open triangle is the activity for the preparation when exposed to saline only. The open circles are the activity immediately after exposure to low pH (5.0) and the open diamonds after 1 hour exposure. Here the later time in displacements gave rise to more activity in general.

Representative responses from a 0.5 sec and 4 sec displacements for saline exposure and after 1 hour exposure to ruthenium red (1mM) as well as after 1 hour exposure to amiloride (1mM) from a preparation are shown in Figure 5. As for the pH exposure, the 0.5 sec bins did show some increases as well as decreases over time, but no

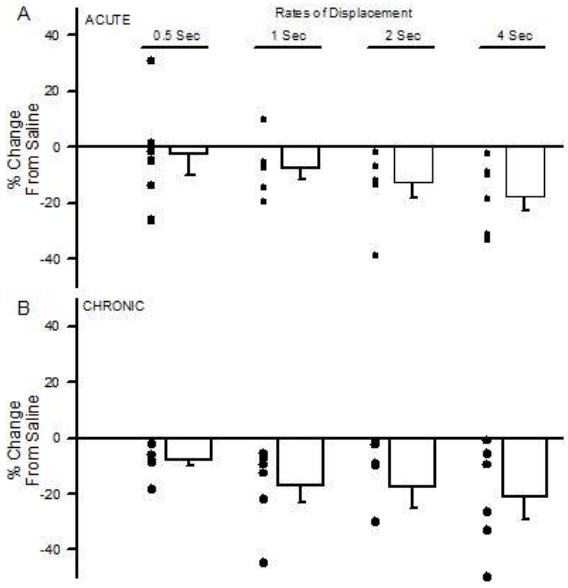

Figure 4: The sensitivity of the crab PD organ to low pH. Percent change in the neural activity is measured by the total number of spikes for the given displacement rates with acute and chronic exposure to low pH. The percent change to exposure of acute and chronic low pH is compared to the initial saline exposure for each displacement rate. The average (+/- SEM) is for six preparations and the closed circles are the values for each individual preparation.

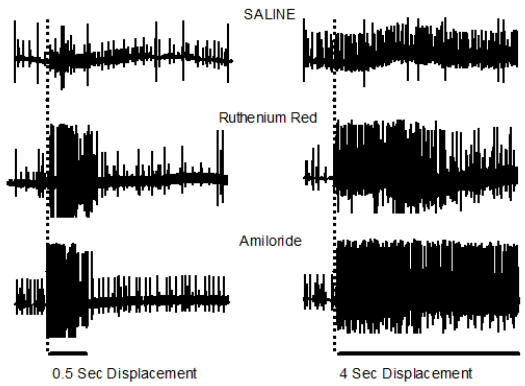

Figure 5: Representatives activity profiles for a PD organ of the crab during exposure to saline and after 1 hour exposure to ruthenium red (1mM) and 1 hour exposure to amiloride (1mM) for the 0.5 and 4sec displacements.

consistent trend for a given time within the displacements is noted for all 6 preparations. So, the total number of spikes is also determined for each displacement time and averaged for all the preparations to determine a percent change from saline to acute and chronic exposure to ruthenium red (Figure 6A) and amiloride (Figure 6B). Since there are no significant differences for acute and chronic exposure and for the different displacements rates, only the chronic exposure is shown in the figure. As with the pH exposure there is a wide range of variation among the preparations, which accounts for the large standard error of the mean. The fact that the preparations are not silenced by either agent indicates the SACs are not blocked by exposure to these compounds.

The low pH, ruthenium red and amiloride did not block or activate the SACs; however, Gd^{3+} (1mM) exposure

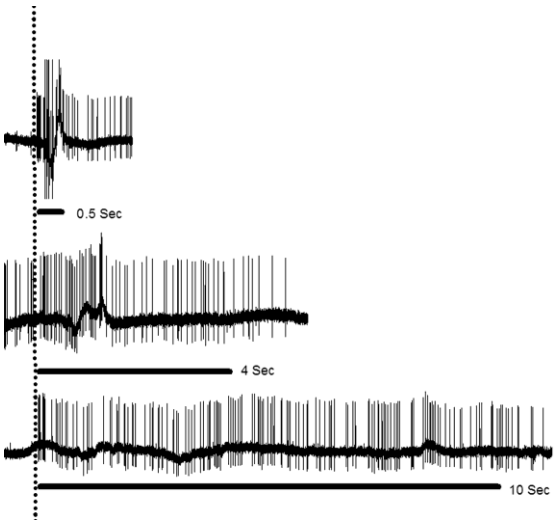

Figure 6: Representative recordings obtained from the crab PD nerve with exposure to the various pharmacological compounds. The mean (+/- SEM) nerve activity for the 0.5, 1, 2 and 4 sec displacements are shown. The total number of spikes produced within the time period of the displacement was determined and used to calculate a percent difference from saline only exposure to that of ruthenium red and amiloride after 1 hour of exposure. The mean changes are greater for amiloride than ruthenium red.

terminated nerve activity after approximately 10 minutes in all preparations (6 of 6; $P < 0.05$ rank sum non-parametric). The Gd^{3+} did not kill the preparations as partial recovery occurred in every preparation with extensive exchanging the bathing medium back to normal saline. The larger amplitude spikes are compromised first, suggesting that the dynamic sensitive neurons are most rapidly affected followed by the static position sensitive neurons (Figure 7).

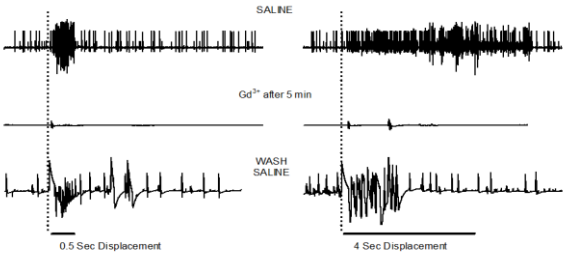

Figure 7: Gd^{3+} exposure to the PD organ of the crab rapidly silenced the neural activity. After exchanging the saline back to saline without the Gd^{3+} the activity would start to return but not as robust as before the exposure. Here only the half and 4 sec displacements are shown as representative responses.

Crayfish MRO

The crayfish MRO shows the same trends as the crab PD organ for the various compounds. Representative responses for the MRO to the displacements are shown in Figure 8. The 0.5 and the 4 sec displacements as well as the stretch and hold position for 10 sec are presented in Figure 9 for a representative preparation with acute and chronic exposure to low pH (5.0). In this particular example, the activity is increased by low pH, but varied for acute and chronic times for a given displacement.

As with the PD preparations, the MROs of the crayfish are exposed to the same concentrations of compounds and for the same amount to time. The 0.5 sec binning of the responses for the 0.5 sec and 4 sec displacements did not show any significant trends. So, the total number of spikes are counted during the displacements and used for determining a percent change in activity when exposed to the various compounds. The variation in the percent changes is substantial among the preparations, which are shown by the large standard errors in Figure 10. There is no

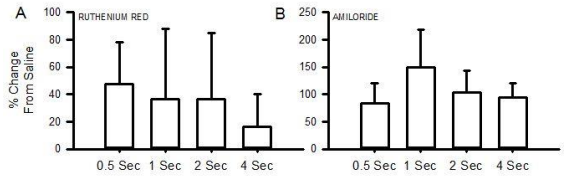

Figure 8: Representative recordings obtained from the crayfish MRO nerve with exposure to the various pharmacological compounds. The nerve activity for the 0.5 and 4 sec displacements, as well as a segment within the 10 sec static hold position, is shown when the preparation is bathed in normal saline.

significant change in activity while exposed to low pH, ruthenium red or amiloride.

However, the nerve activity is terminated when exposed to Gd^{3+}, as with the crab PD preparations.

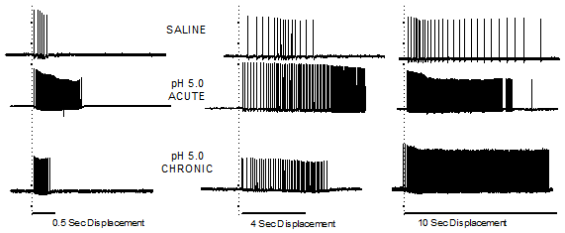

Figure 9: The activity for the crayfish MRO from a representative preparation when exposed to saline and then low pH (acute) and after 1 hour exposure (chronic) to the low pH for half and 4 sec displacement of the joint as well as holding the joint bent for 10 sec.

Discussion

In this study we demonstrated that the firing rates of the PD and MRO over a physiological range of movements did not show sensitivity to two common SAC

blockers (amiloride, ruthenium red) nor was there a significant increase in activity due to

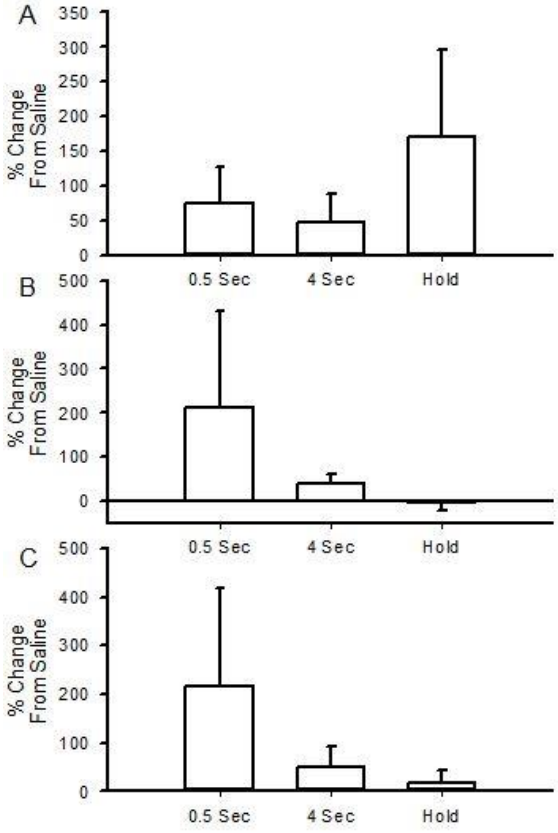

Figure 10: Quantifying the neural activity over the various displacements utilized for the crayfish MRO. (A) The number of spikes which occurred for the total time of the displacements (0.5, 4 sec and hold) during exposure to saline, low pH, ruthenium red as well as amiloride was counted and a percent change to saline exposure was determined. The average percent changes (+/-SEM) to the displacements for low pH (A), ruthenium red (B) and amiloride (C) after 1 hour of static exposure are shown.

H^+. However, Gd^{3+} did have rapid effects in shutting down activity. This would suggest that the SAC within the COs and MRO are not of the DEG/ENaC channel subtype. Since relatively high concentrations of amiloride (1mM), ruthenium red (1mM), and H+ (pH 5.0) were used, it is likely the SACs in the sensory endings are not affected by these agents. Given Gd^{3+} at 1mM blocked the activity rapidly, it is suggestive that access of the other agents is not an issue. We would suggest to refer to the SACs within these COs as amiloride, ruthenium red and acidic insensitive channels. Since Gd^{3+} is a broad non-selective inhibitor of SACs, it was not surprising that the SAC in the neurons of these COs were blocked.

The mechanisms of blocking SACs from a closed or open state would not be a factor here as the neurons were repeatedly activated in order to elucidate sensitivity to each SAC blocker. In addition, the amiloride and ruthenium red bathing solutions were exposed to the preparations for an hour without any significant decrease in activity upon CO activation. The long incubation times and maintained activity is also an indication of the robust nature of these invertebrate preparations. The rapid diffusion of H^+ due to its small size and charge allows access to the SACs in the sensory endings. The finding that an extracellular pH_o of 5.0 had no substantial effect on activity is fairly surprising as one might have expected some potential influence on the voltage gated Na^+ and K^+ channels. In rodents, pH_o of 6.4 resulted in a reduction of more than 10% of the peak voltage gated Na^+ current (Tombaugh and Somjen, 1996). The voltage gated Na^+ and K^+ currents in squid are also decreased by low pH_o (Stillman et al., 1971; Carbone et al., 1978). However it is known species differ, even among invertebrates, on the influence of Na^+ and K^+ channels by low

pH_o. As compared to the squid, 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 (Shrager, 1974). 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 MRO has been a model preparation to address sensory transduction for a number of years (Erxleben, 1989), but the SACs have not been fully characterized pharmacologically or at a molecular structural level. Voltage-clamp and patch-clamp studies of the neuronal membrane have provided a fundamental understanding of ionic flow, channel distribution, and density, as well as regional properties of sensory neurons (Brown et al., 1978; Hunt et al., 1978; Edwards et al., 1981; Erxleben, 1989; Rydqvist and Purali, 1991; Rydqvist and Swerup, 1991; Purali and Rydqvist, 1992). There does not appear to be a Ca^{2+} influx responsible for the action potential in the axons of the neurons associated with the MRO (Erxleben, 1989; Purali and Rydqvist, 1992). This is pertinent, as Gd^{3+} is known to block voltage gated Ca^{2+} channels and if they are not prevalent on these axons it would appear Gd^{3+} was targeting the SACs in the sensory endings. Then this would depress an action potential from being generated. To address this with confidence, one could record the graded sensory potentials in the cell body, which is close to the sensory ending before and after application of Gd^{3+}.

Both neurons rapidly shut down in the presence of Gd^{3+}. The crab PD CO is very different than the MRO, in that the sensory endings are not embedded in a muscle fiber. Instead, they are located in an elastic strand which when stretched causes the sensory endings within the scolopale caps to detect the displacement (Whitear, 1960, 1962, 1965). The scolopale caps are the locations in which the sensory endings are embedded. It is known that action potentials are initiated in the sensory endings within the scolopidium (Mendelson, 1963; Hartman & Boettiger, 1967), and, therefore, the transduction process is located in the endings. The COs in the crab are similar anatomically to those in joints of insects. The number of sensory neurons within the PD nerve will vary depending on the age of the animal (Cooper and Govind, 1991; Hartman, and Cooper, 1994). For the size of the animals used in this study, for what appeared to be non-regenerated legs, we estimate there are about 80 neurons with the majority being ones to monitor static positions of the joint (Cooper and Govind, 1991; Hartman, and Cooper, 1994; Majeed et al., 2013). This is the reason for so many different sized extracellular spikes in the nerve recordings. The PD preparation is even more robust than the MRO for maintaining recordings over time in minimal saline as the PD organs can show consistent responses after being in saline for 4 to 6 hours, whereas the MRO preparations show a rundown in responsiveness after 3 hours (personal experiences in the teaching lab). This is likely due to the sensory endings not being directly associated with muscle fibers. The smaller cell bodies and the smaller associated axons in the PD organ are of the static position sensory cells, and the larger cell bodies (with larger axons) are neurons which detect the dynamic movements of the strand (Cooper, 2008; Majeed et al., 2013). The smaller extracellular spikes from the static position sensitive neurons drop out quicker than the larger spikes from the dynamic neurons with treatment of Gd^{3+}. The large cell bodies of the two neurons associated with MRO are substantially larger than those associated with the crab PD; however, the sensory endings are embedded within the muscle fiber which

may make access to exogenous agents more difficult. The ultrastructure of the sensory endings for the COs reveal the endings are paired within a scolopale that itself is embedded within the elastin and collagen tissue of the chordotonal strand (Whitear, 1962). However, it appears compounds are readily accessible to the endings since Gd^{3+} rapidly blocked the responses. The MRO required a few more minutes to show a similar effect to Gd^{3+}, which is likely due to the sensory free endings spread out within the muscle fibers (Florey and Florey, 1955). The muscle fibers of the MRO are much smaller in diameter then the neighboring fibers of the other abdominal skeletal muscles, except the small fibers of the dorsal membrane muscle and the superficial extensor muscle accessory head (Sohn et al., 2000). The cell bodies of the MROs are very accessible to applied compounds, but the targeted channels on the sensory endings for the mechanical transduction are less so.

Perhaps the SACs in insect COs are of a similar protein make up and also show insensitivity to amiloride, ruthenium red, and H^+ (pH 5.0), just as in the crab. The mechanosensitive neurons (i.e., dorsal bipolar dendritic neurons), which detect deformation cuticle in *Drosophila* do appear to be amiloride sensitive and are of the Piezo subset (Suslak et al., 2015); however, as far as we know, the neurons for COs associated in the limbs of insects have not been carefully examined for their pharmacological properties. Although, various insecticides have been tested and only a few synthetic ones appear to be selective to COs. Pymetrozine seems to be selective in targeting COs associated with the wing hinge stretch receptor (Ausborn et al., 2005). Studies are currently underway within our group to examine this insecticide and other compounds on crustacean sensory receptors, particularly those associated with the COs in the limbs.

These experiments have helped in further classifying the subtypes of SACs in proprioceptive neurons associated with COs of crustaceans and the MRO of the crayfish. Even though amiloride and ruthenium red did not block, and low pH did not stimulate the SACs in these structures, the newly acquired knowledge aids in understanding that these mechanoreceptors are not of the family that are targets of these compounds. Knowing that the non-selective blocker Gd^{3+} does rapidly block responses helps confer that there are at least some similarities to other known SACs. Since COs are ubiquitous within arthropods, and the crayfish MRO has a counterpart within mammals (muscle spindles), the more we learn about how sensory endings transduce the mechanical signals in these invertebrates, the more we enrich our knowledge regarding the variety of SAC subtypes in general. (Kavlie and Albert, 2013; Boscardin et al., 2016). This comparative approach taught us about the similarities and differences in mechanosensory proteins from pharmacology to potential genomic variances. Future studies would be beneficial in addressing the ionic currents through the SACs and performing a protein sequence analysis of the SACs in these preparations.

Endnotes

1. Many of the authors were students in a neurophysiology lab based class who addressed authentic scientific based questions in regards to the topic of examining pharmacological agents known in other models to block stretch activated ion channels. This course project is part of a new trend in teaching science to undergraduates (Linn et al., 2015). Course-based undergraduate research experiences

(CUREs) are relatively new and an approach being adopted by science educators in high schools and colleges (Bakshi et al., 2016).

Acknowledgments

This work was funded by student laboratory fees for a course taught in Department of Biology, University of Kentucky and personal funds (RLC). The course is neurophysiology lab (Bio446, Bio650).

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