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Article

The Effect of Calcium Ions on Mechanosensation and Neuronal Activity in Proprioceptive Neurons

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Abstract: Proprioception of all animals is important in being able to have coordinated locomotion. Stretch activated ion channels (SACs) transduce the mechanical force into electrical signals in the proprioceptive sensory endings. The types of SACs vary among sensory neurons in animals as defined by pharmacological, physiological and molecular identification. The chordotonal organs within insects and crustaceans offer a unique ability to investigate proprioceptive function. The effects of the extracellular environment on neuronal activity, as well as the function of associated SACs are easily accessible and viable in minimal saline for ease in experimentation. The effect of extracellular $[Ca^{2+}]$ on membrane properties which affect voltage-sensitivity of ion channels, threshold of action potentials and SACs can be readily addressed in the chordotonal organ in crab limbs. It is of interest to understand how low extracellular $[Ca^{2+}]$ enhances neural activity considering the SACs in the sensory endings could possibly be Ca^{2+} channels and that all neural activity is blocked with Mn2+. It is suggested that axonal excitability might be affected independent from the SAC activity due to potential presence of calcium activated potassium channels ($K_{(Ca)}$) and the ability of Ca^{2+} to block voltage gated Na^+ channels in the axons. Separating the role of Ca^{2+} on the function of the SACs and the excitability of the axons in the nerves associated with chordotonal organs is addressed. These experiments may aid in understanding the mechanisms of neuronal hyperexcitability during hypocalcemia within mammals.

Keywords: crab; sensory; stretch activated channels; nerve; calcium; manganese

1. Introduction

It is well established that free Ca^{2+} within the presynaptic terminal is key for chemical synaptic transmission [1,2] and is also important for electrical transmission though gap junctions [3]. However, the impact of ionic Ca^{2+} on electrical conduction along neurons remains to be addressed. The role of Ca^{2+} on the generation of action potentials, conduction of electrical events as well as other biophysical properties of the membrane differs among neurons within an animal and among animals. In mammals, hypercalcemia as well as hypocalcemia is life threatening [4] because of the effects on function of the cardiovascular system, neurons as well as synaptic transmission. To address the effects of Ca^{2+} on intact neural circuitry, brain slices of rodents [5,6] has been used where most of the attention is focused on the impact on synaptic transmission [2,7,8] and ionic currents in single

cells [9]. It still remains to understand how the [Ca^{2+}] impacts conduction along central neurons. There are two common responses in humans which are indicators of someone in a state of hypocalcemia where motor nerves are activated to result in twitches of the face (Chvostek's sign; [10]) or a slow contraction of the muscles in the hand resulting in flexion referred to as carpopedal spasm (Trousseau's sign; [11]). However, there is some doubt the Chvostek's sign are all related to hypocalcemia [10,12,13]. These signs have led to much speculation as to the mechanism of the responses due to low extracellular Ca^{2+} which appears to be counterintuitive due to the role of Ca^{2+} in enhancing synaptic transmission at the neuromuscular junction [1,2]. The mechanisms behind these observed phenomena are still debated as to the effects of excitability, conduction of electrical signals, the types of ion channels activated or inhibited by Ca^{2+} in neurons [14–19].

Since altered levels of [Ca^{2+}]$_o$ can impact neuronal function independent of synaptic transmission, it is of interest to know the effects on sensory perception and conduction to the CNS before central processing. In addressing a given sensory system one can address fundamental aspects of the effects of altered [Ca^{2+}]$_o$, without the involvement of synapses, which may well be applicable to other types of neurons within a neural circuit.

In addressing the role of Ca^{2+} in propagation of electrical signals of neurons there are a few potential explanations for the observed effects. There is the possibility of Ca^{2+} ions altering the screening charge of the membrane and associated proteins (i.e., channels) [20], Ca^{2+} ions blocking voltage-gated ion channels and altering the kinetics of the channels [9,17,21] and activation of calcium activated potassium channel ($K_{(Ca)}$) [17]. One or in combination of these possibilities can influence excitability, conduction properties and refractory period of the neurons. Isolated nerves allow one to address these possibilities but usually a nerve used as a model is a mix of neuronal types (sensory and motor) and of a mix in types of myelinated and unmyelinated neurons which can induce other variables needing to be considered. In developing neuronal models for addressing the effects of extracellular Ca^{2+} on the biophysical properties of neurons and on a given sensory modality, we used a chordotonal organ in the limb of the marine crab which monitors the proprioceptive status of the distal joint in walking legs [22]. These neurons are all sensory and unmyelinated. Using this preparation, one can address the effect of Ca^{2+} on the mechanosensitive channels (i.e., stretch activated channels, SACs) as well as for propagation of the nerve signals. The mechanosensitive channels for proprioception in mammals have recently been identified as PIEZO2 subtype in muscle spindles [23]. To date, the subtype of SACs in chordotonal organs in crustaceans has not been fully identified. It appears they are not PIEZO1 nor DEG/ENaCs (Degenerin/epithelial sodium channel) subtype from previous studies as examined with low pH (pH 5), ruthenium red, amiloride and Gd^{3+} [24–26]. Only Gd^{3+} provided evidence of reduced activity and was reversible but it is not a specific blocker in types of SACs [26]. The SACs might be a type of Ca^{2+} ion channel, but this has yet to be determined. In addition, unlike the muscle spindles in vertebrates, the sensory endings of the chordotonal organs are not embedded in muscle fibers. The contraction of the intrafusal muscle fibers in the muscle spindle is altered by differing concentration of extracellular calcium [Ca^{2+}]$_o$ and the effect on the membrane potential of the muscle can impact the SAC in the sensory endings. The arrangement the crab chordotonal organ enables for the exploration of the direct effects of agents on the sensory endings, without the effects on muscle fibers, as well as axon excitability [27,28]. Given there is still an active interest in how [Ca^{2+}]$_o$ influences the function of nerves as well as understanding the function of SACs, in the vast variety of SAC subtypes, among organisms [29], the crab leg proprioceptive neurons serve as a viable model for such investigations [24–28,30].

2. Materials and Methods

2.1. Animals

Blue crabs (*Callinectes sapidus*) were obtained from a local supermarket in Lexington, KY, which were delivered from a distribution center in Atlanta, GA. They were bought and maintained in a seawater aquarium for several days prior to use to assess their health. The

crabs were adults and in the range of 10–15 cm in carapace width (from point to point). All crabs used were very active upon autotomizing a leg for experimentation.

2.2. Dissection and Physiology

Similar dissection procedures and electrophysiological measures as is described in detail in text and video format [22]. In brief, the animal was induced to autotomize the first or second walking leg by lightly pinching with pliers at the base of the leg. The propodite-dactylopodite (PD) chordotonal organ spans the last segment of the leg and was exposed through cutting a window of the cuticle on both sides of the leg (in the propodite segment; Figure 1A). With a window in the cuticle the PD nerve can be observed independent of the main leg nerve (Figure 1B). The chordotonal organ spans the PD joint and the neurons in various regions of the PD organ (Figure 1C). After physiological recordings were made the preparation was stained with methylene blue to observe the PD nerve (Figure 1D) and the cell bodies of the sensory neurons (Figure 1E). Staining with methylene blue is also useful to learn the dissection so the physiology can be accomplished without staining.
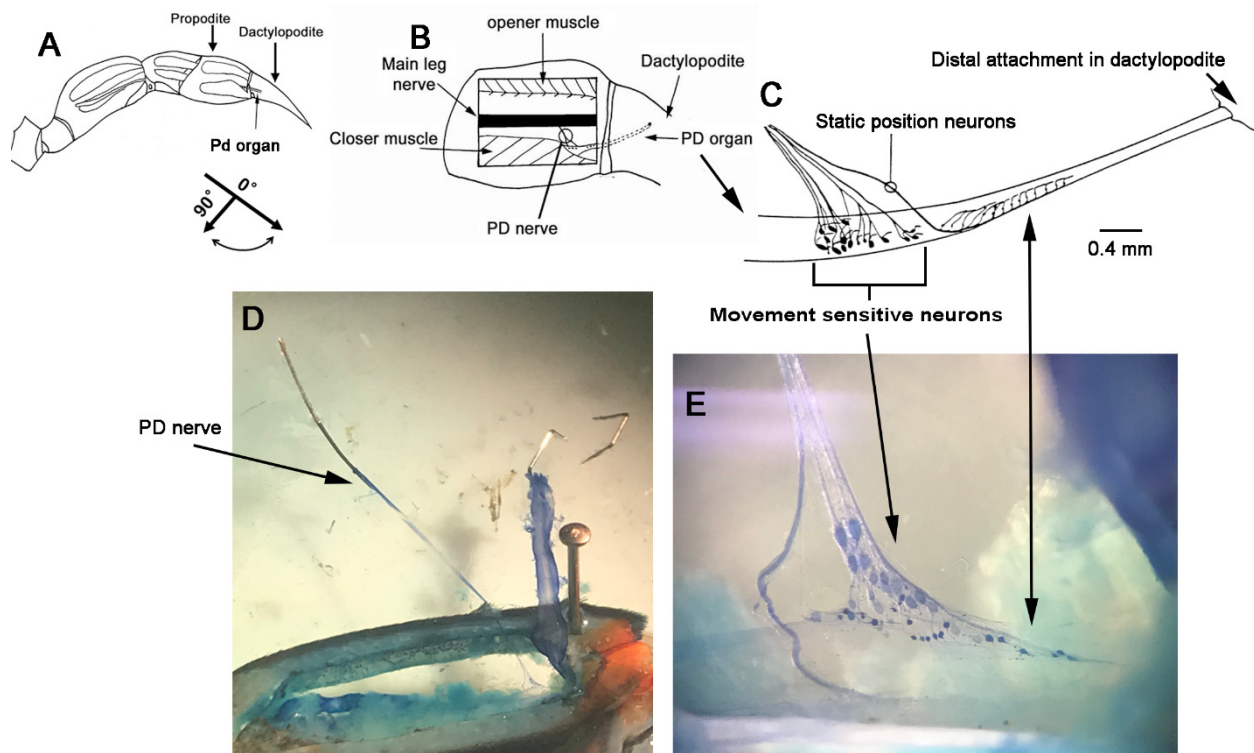
Figure 1. The isolation of the PD nerve for electrophysiological recordings. (**A**) The PD organ spans the most distal joint in the limb between the propodite and dactylopodite. (**B**) The PD nerve branches away from the main leg nerve close to the base of the chordotonal strand. (**C**) The PD nerve contains neurons which monitor movement as well as static positions of the chordotonal strand. (**D**) The PD nerve can carefully be dissected away from the main leg nerve for electrophysiological recordings. (**E**) In learning the dissection and to observe the individual neurons, the preparation was stained with methylene blue.

The leg was pinned in a Sylgard-lined dish and covered with crab saline (Figure 2A). The PD nerve was then exposed and pulled into a suction electrode for recording. During the experiment, the dactyl was moved from a flexed position to an open position in a 1 s time frame, held for 10 s and then moved back to the starting position (Figures 1A and 2A). An insect dissecting pin was used to mark the maximum displacement range for consistency among trials and each displacement was marked on the recorded computer file. The PD nerve can be carefully dissected away from the main leg nerve for varying lengths. The proximal end was pulled into a suction electrode to record nerve activity.

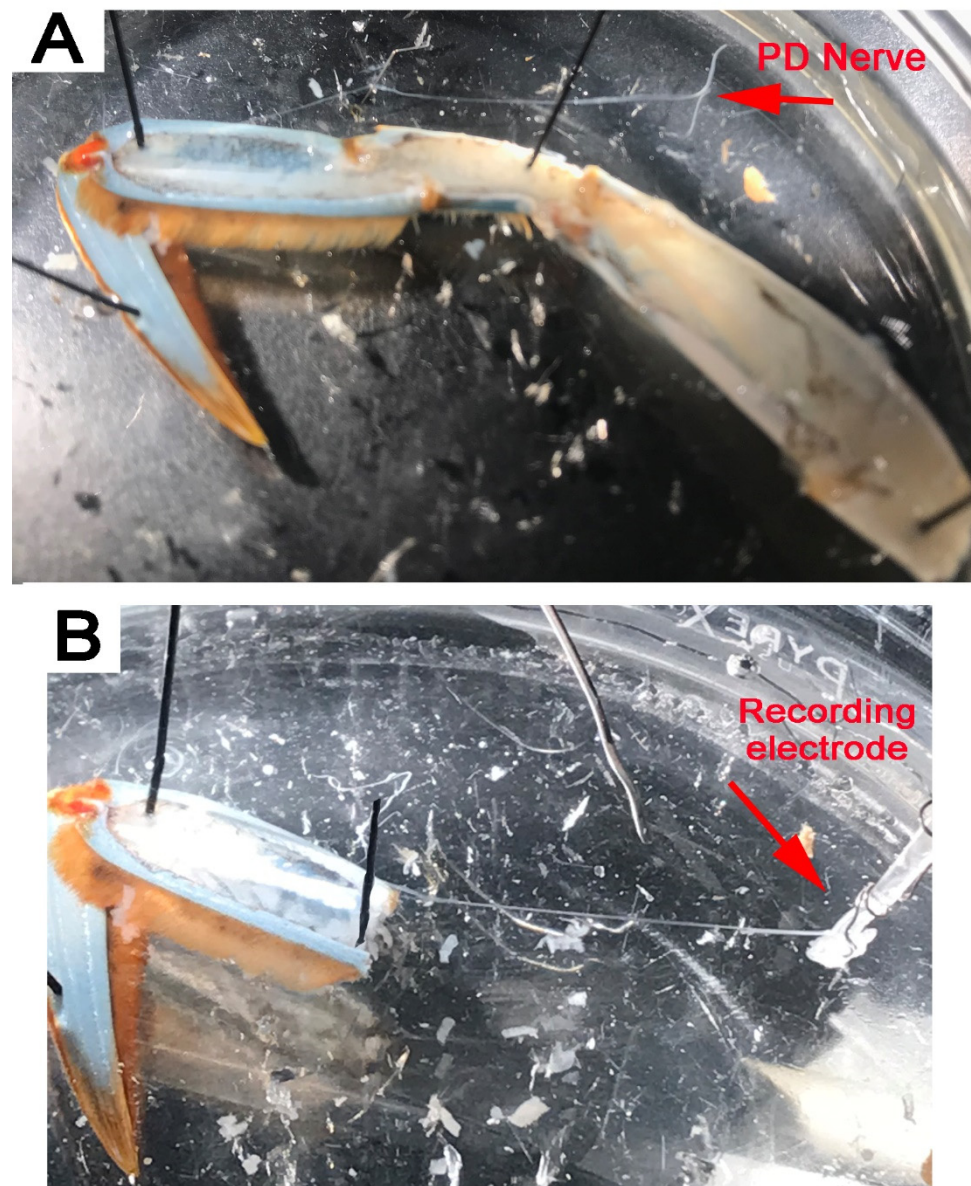**Figure 2.** Exposing the PD nerve and distal limb segments for recording nerve activity. (**A**) A length of PD nerve can be isolated from the main leg nerve for the length of the limb from the autotomy plane. This dissection can be used for monitoring joint movement as well as inducing and recording compound action potentials of the PD nerve. (**B**) Isolating the leg segments not needed and keeping the two most distal segments with the long length of the PD nerve, reduced movement artifacts of touching the recording electrode with the cuticle when moving the joint. This also helped to maintain the saline integrity from damaged tissue within the exposed leg.

The PD nerve was left intact to the PD organ to record the activity from static position neurons, with the joint pinned in a set position (Figure 3). In this arrangement compound action potentials (CAPs) are initiated by stimulating the proximal end of the PD nerve isolated from the main leg nerve or with leaving the main leg nerve associated with the PD nerve. Leaving the main leg nerve associated helped to pin the connective tissue and blood vesicle to the dish and sped up the dissection. The PD nerve is well exposed to compounds while changing out the bathing media when the main leg nerve is left with the PD nerve. The PD nerve was cut away from the PD organ when recording isolated CAPs without the background firing from the static position neurons. The isolated PD nerve was used for recording the refractory periods of the PD nerve in the varying conditions.
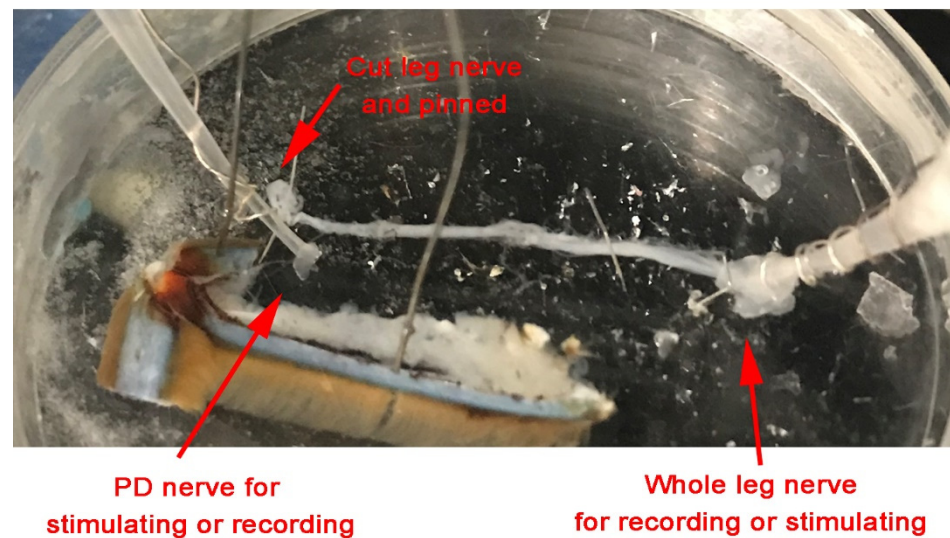
Figure 3. Set up for recording sensory activity of the PD organ and compound action potentials (CAPs) of the PD nerve. Leaving the PD nerve intact along the main leg nerve decreased the dissection time and reduced the likelihood of damaging the PD nerve. The distal end of the main leg nerve can be cut more distal than where the PD nerve branches off. This provided tissue to pin the main leg nerve to the dish and provide slack in the PD nerve for en passant recording or stimulating for measuring CAPs. The proximal end of the main leg nerve was used to record or provide stimulation for inducing CAPs. In this arrangement the activity of static-position sensitive neurons or activity from joint movement while inducing CAPs can also be monitored along with CAPs.

The standard crab saline used during recordings of the sensory nerves consisted of (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 ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) acid and 5 HEPES base adjusted to pH 7.5. Each concentration of $CaCl_2$, $BaCl_2$ and $MnSO_4$ was examined for 6 preparations. The various concentrations of salts and compounds are presented along with the results for the activity measures. As a control for higher salt concentrations in the bathing media, sucrose or mannitol was used.

The numbers of extracellular recorded action potentials (i.e., spikes) recorded over the first 10 s from the start of the joint displacement were used as an index in the neural activity. In each bathing condition, the joint displacement occurred three times with 10 s between displacements. The number of spikes of the three trials was averaged for graphical purposes and comparisons among bathing conditions (Figure 4).

2.3. Statistical Analysis

All data are expressed as a mean ($\pm$SEM-standard error of the mean). The paired *t*-test was used to compare the differences in responses before and after exchanging solutions. Normality test was the Shapiro–Wilk. Wilcoxon rank sum, non-parametric test was used when appropriate as presented with the results. To compare among different conditions an all pairwise multiple comparison procedure with a Tukey Test was used or a Friedman repeated measures analysis of variance on ranks along with a one-way repeated measures analysis of variance and a post analysis with an all pairwise multiple comparison procedure with a Bonferroni *t*-test. The analysis was performed with Sigma Stat software. A *p*-value < 0.05 was considered statistically significant for determining changes for exposure to the compounds. To examine the consistency and reproducibility of the data, groups of participants blinded to the specific settings of the analysis software were asked to supply their interpretations of the number of spikes for some of the same data sets.
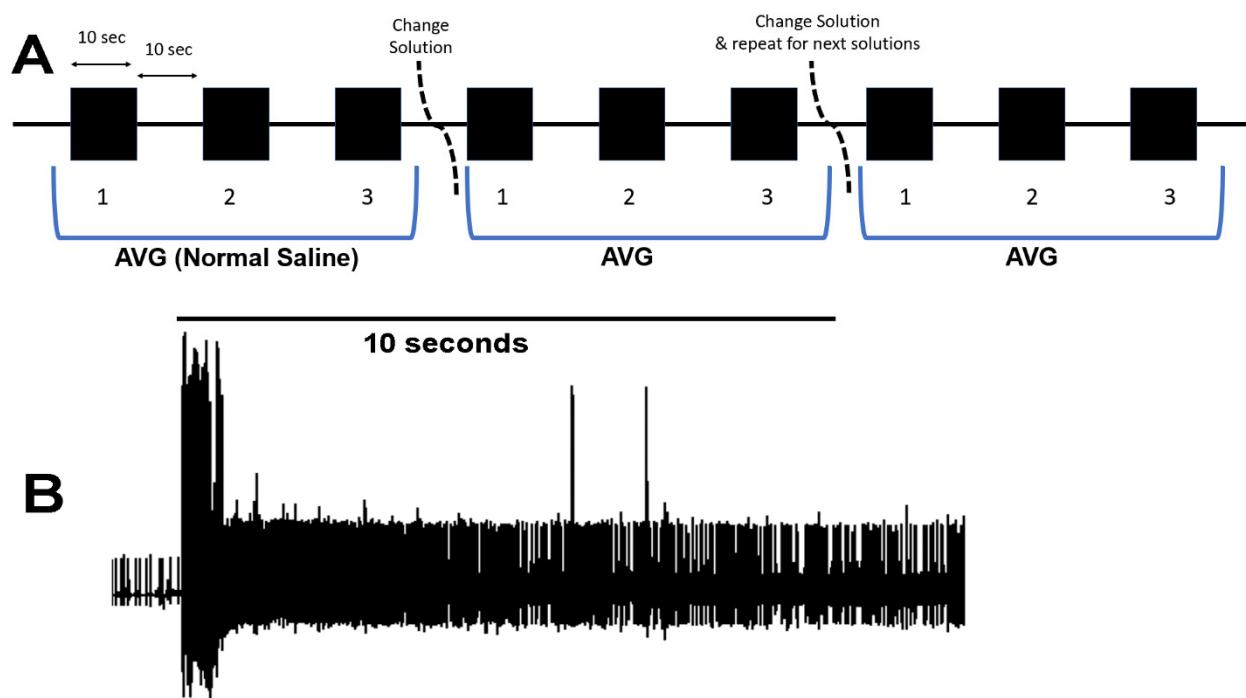
Figure 4. The experimental paradigm in joint displacements and analysis of the spikes. (**A**) The joint is displaced from a flexed position to an extended position within 1 s and held in extended position for at least 10 s and then moved back to a flexed position. This was repeated three times for each bathing solution. Depending on the media used the preparation was bathed for a given amount of time. The joint was rapidly moved back and forth 10 times after each exchange in the media (not illustrated in figure) and then the three trials from a flexed position to an extended position are repeated. (**B**) The number of spikes obtained from the beginning of the movement (1 s) and over the held static position for the next 9 s was used as an index of the neural activity of the PD organ. AVG means an average of the three trials is used for an index in a given condition.

3. Results

3.1. Altering Extracellular [Ca^{2+}] Effects on Proprioceptive Activity

The effect of altering [Ca^{2+}] on the function of the chordotonal organ and nerve activity was performed by first bathing the preparation in standard saline with the standard [Ca^{2+}] of 15 mM and displacing the joint to obtain a baseline activity profile for the preparation. Then, the bath was exchanged with varying [Ca^{2+}] and the same displacements were performed. To determine if altered activity compromised the preparation (i.e., damaged the neurons) the bath was exchanged back to the standard saline and activity was again obtained with joint displacements. Standard saline of 15 mM Ca^{2+} was used as a control for exchanging the bathing media and repetitive displacements. Representative traces for each of the bathing conditions are shown in Figure 5.

The average number of spikes of the three trails in each bathing media was used as an index of neural activity. The responses due to the joint displacements from each preparation while bathing in varying [Ca^{2+}]$_o$ is illustrated in Figure 6. The exposure to 45 mM or 100 mM Ca^{2+} significantly decreased responsiveness to joint displacement as compared to the initial saline exposure of 15 mM Ca^{2+} (Paired *T*-test, $p < 0.05$, $N = 6$ and/or 7 for each condition). In contrast, a saline in which no Ca^{2+} was added increased responsiveness to joint displacement as the average number of spikes increased significantly compared to the initial saline exposure of 15 mM Ca^{2+} (Paired *T*-test, $p < 0.05$, $N = 6$ and/or 7).
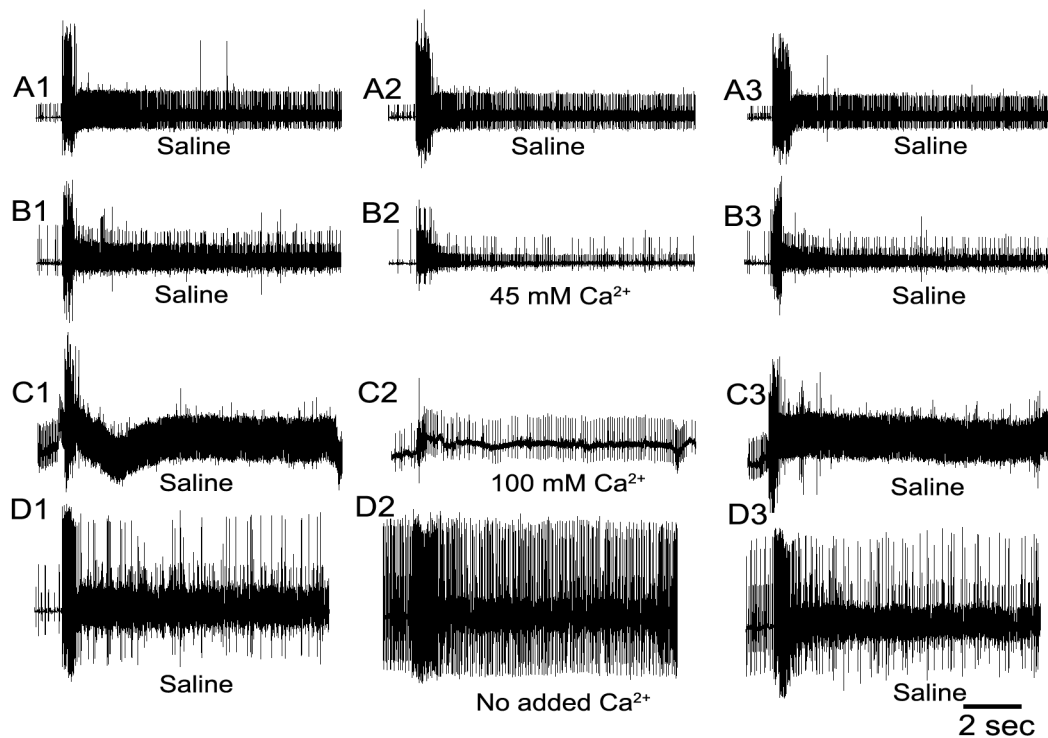
Figure 5. Representative recordings of one trail of three in each of the varied conditions in exchanging the bathing media with alterations in the extracellular [Ca^{2+}] on the activity of the PD organ during displacements. For each preparation the activity in the initial standard saline (15 mM Ca^{2+}) is shown. Then, after the bath was changed with (**A**) control (i.e., standard saline) or (**B**) 45 mM or (**C**) 100 mM or (**D**) no added Ca^{2+}. Each condition is also shown with a return to normal saline after flushing the recording chamber.

The standard saline contains 6.98 mM Mg^{2+} and could potentially aid more in blocking SACs in the sensory endings with the absence of Ca^{2+}. That is if Ca^{2+} was having a direct effect on the function of SAC. However, in examining five preparations in which the bathing media was exchanged from standard saline to one in which Mg^{2+} and Ca^{2+} was not added, the results did not have a consistent trend (Figure 7). As shown, one preparation had a large increase in activity with joint displacement for the saline without Mg^{2+} and Ca^{2+} while others decreased in the activity. These results are important as this indicates the SACs themselves can function without Ca^{2+} and Mg^{2+} added to the saline. In addition, just as important is knowing that the nerve can conduct the electrical signals without Ca^{2+} and Mg^{2+} in the media.

In order to understand how another divalent cation (i.e., Mn^{2+}) influences activity of the SACs and electrical conduction along the nerve, a high concentration of Mn^{2+} was exposed to the preparation while the joint was held in a static position. In addition, the PD was stimulated to evoke compound action potentials (CAPs) while monitoring the activity from the static position sensitive-neuronal firing. The exchange in the bathing media from standard saline to one containing Mn^{2+} (100 mM) depressed the responses from the static position-sensitive neurons (6 out of 6 preparations, *p* < 0.05 Wilcoxon rank sum, non-parametric test; Figure 8). Interestingly, the CAPs were able to be maintained suggesting the mechanism of action of Mn^{2+} depressing activity is at the level of the SACs.
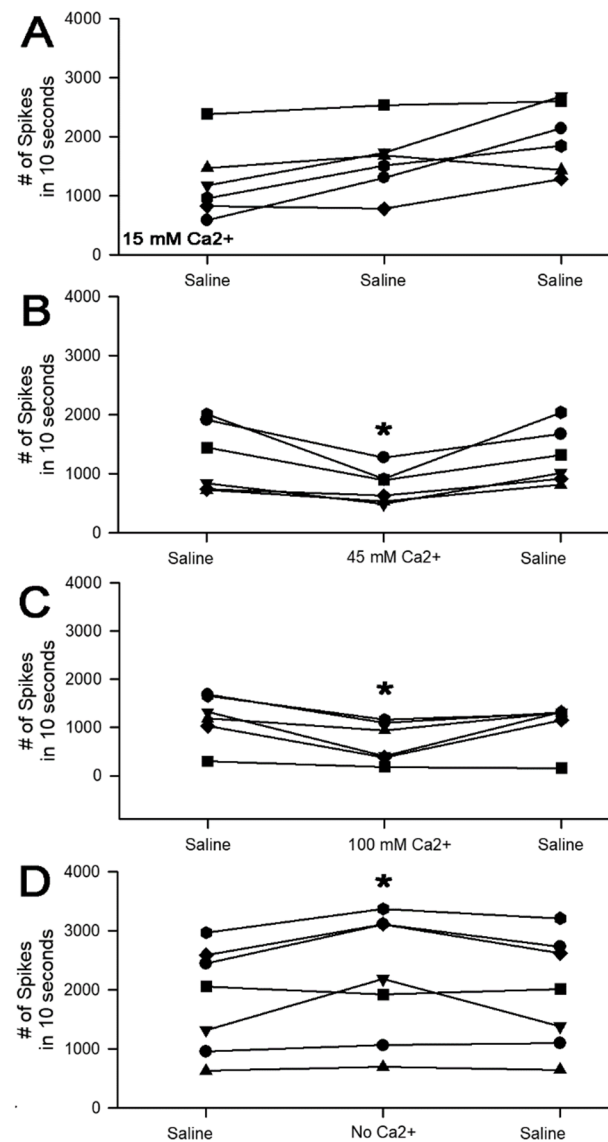
Figure 6. The activity profiles for each preparation in the paradigms of altering the $[Ca^{2+}]$ in the bathing media. The activity in (**A**) standard saline (15 mM Ca^{2+}) (**B**) 45 mM or (**C**) 100 mM or (**D**) no added Ca^{2+} is shown. Both conditions of 45 mM and 100 mM Ca^{2+} decreased activity, while the saline with no added Ca^{2+} increased activity. The comparisons are made to the initial saline exposure for each preparation (Paired *T*-test, $p < 0.05$, $N = 6$ or 7 for each condition). Each line is an individual preparation. Each symbol is an average number of spikes in the three trials for each condition within the paradigm of changing the bathing media.

To assess the effect of low Ca^{2+} in increasing activity of the PD organ during displacements, the PD was pinned in a static position to monitor the activity profile from the static position-sensitive neurons while at the same time being able to evoke CAPs. In a static position, the exchange of the bathing media to one with no Ca^{2+} presented with an increase in activity in 6 out of 6 preparations ($p < 0.05$ Wilcoxon rank sum, non-parametric test; Figure 9). The evoked CAPs were similar in shape for the two conditions but isolated CAPs without the activity from the organ were difficult to obtain. Thus, the need to cut away the PD organ and to investigate the CAPs with an isolated PD nerve as shown below.
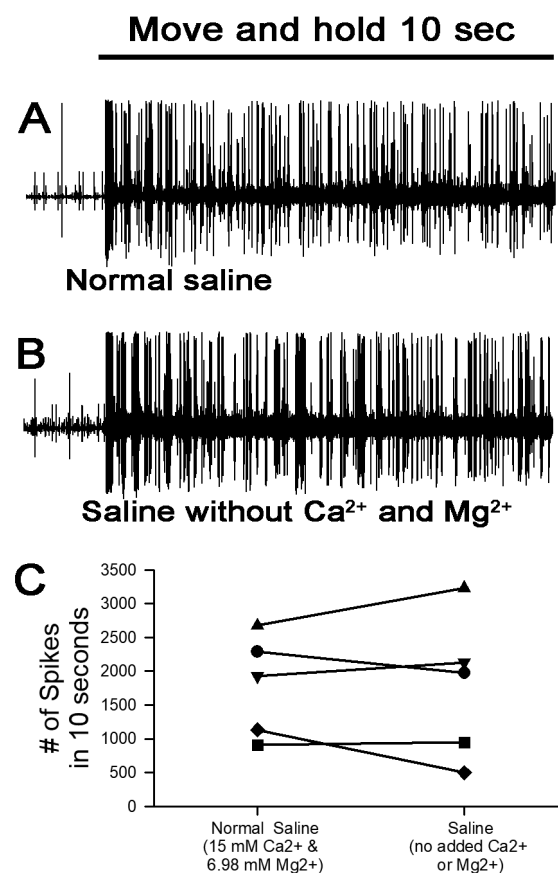
Figure 7. PD organ activity without Ca^{2+} and without Mg^{2+}. A representative activity profile during joint displacement for (**A**) standard saline and after exchanging saline with one where (**B**) $CaCl_2$ and $MgCl_2$ was not added. (**C**) When saline without $CaCl_2$ and $MgCl_2$ was exchanged for the standard saline activity, the neural activity was not consistently increased or decreased with joint displacement.

The isolated PD nerve provided an approach to directly examine the effect of altered $[Ca^{2+}]$ on excitability of the neurons and the shape of the evoked CAPs without activity from activating the SACs. To determine if the amplitude of the CAP would increase, a maximal amplitude was obtained with increasing the stimulus voltage and then decreasing the voltage to produce a submaximal amplitude (Figure 10A). The submaximal stimulus was given while changing the bathing saline to one of no Ca^{2+} to higher $[Ca^{2+}]$. When saline with no added Ca^{2+} is exposed to a nerve being stimulated every 2 s to obtain evoked CAPs, the nerve produces all different shapes and amplitude CAPs. This implies that various groups of neurons are excited at different times (Figure 10B). Upon returning to standard saline the random activity goes away (Figure 10C). Further exchanging of the saline bath for one with high $[Ca^{2+}]$ at 100 mM the CAPs are not able to be induced at the same stimulus voltage, but the nerve requires a more intense voltage to recruit the neurons to be excited (Figure 10(D1–D3)). Returning the saline bath back to standard saline composition the CAPs were still altered in shape after 10 min (Figure 10E). Perhaps more flushing of the bath and a longer wait period is required for activity to return to baseline conditions.
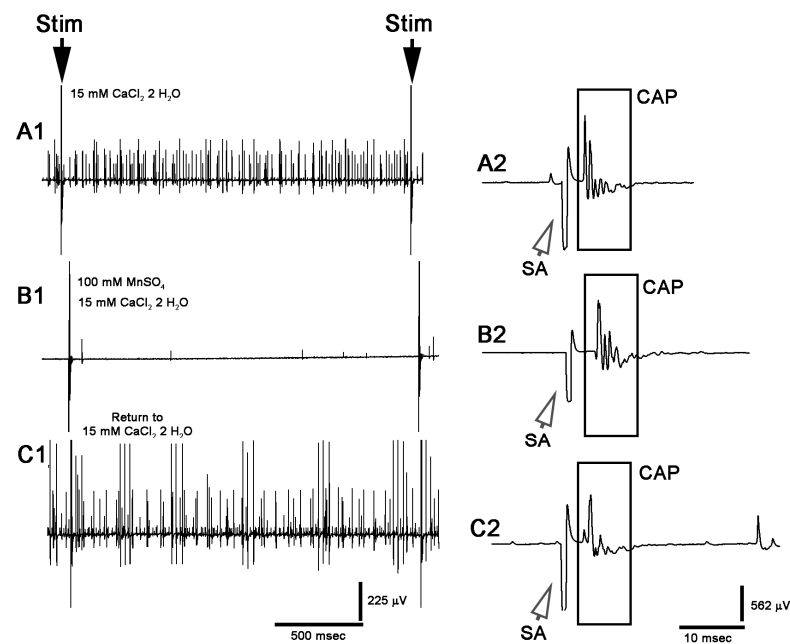
Figure 8. Exposure to Mn^{2+} blocks the SACs but not nerve conduction. (**A1**) With the PD joint held in a static position the static position-sensitive neurons were active in standard saline but were depressed in saline containing (**A2**)100 mM $MnSO_4$. Compound action potentials (CAPs) could be initiated by evoked stimuli of the PD nerve in saline (**B1**) and in the presence of (**B2**)100 mM $MnSO_4$. Upon flushing out the $MnSO_4$ with standard saline the SACs were again activated (**C1**) and the CAPs were still able to be induced (**C2**).
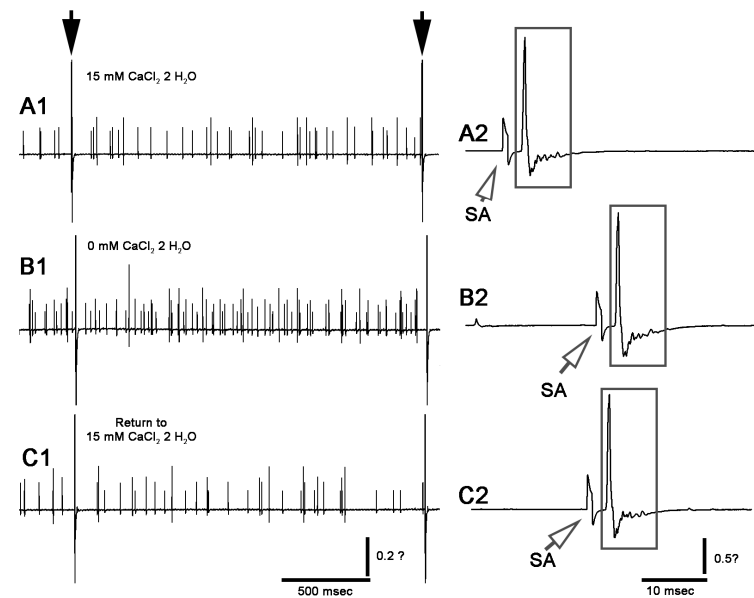
Figure 9. Exposure to reduced Ca^{2+} increased activity of the PD nerve when the PD joint was held in a static position. (**A1**) The activity of the PD organ in standard saline. (**B1**) The activity of the PD organ in standard saline when Ca^{2+} was not added to the saline. (**C1**) The activity of the PD organ upon returning to standard saline. Note the PD organ activity was increased in the saline lacking Ca^{2+} for the joint pinned to the dish in the same position as shown for the activity in A1. The compound action potentials (CAP) initiated by evoked stimuli of the PD nerve in saline (**A2**), in saline without Ca^{2+} (**B2**) and when returned to standard saline (**C2**). Note in B2 there was a spontaneous CAP early in the trace. The random occurrences of CAPs are present when exposed to saline with reduced $[Ca^{2+}]$.

Figure 10. Exposure to reduced [Ca^{2+}] increased excitability of the PD nerve isolated from the PD organ and SACs, while increased Ca^{2+} decreased excitability of the isolated PD nerve. (**A**) The maximum sized compound action potential (CAP) was determined by increasing voltage. The voltage was reduced to be submaximal (red 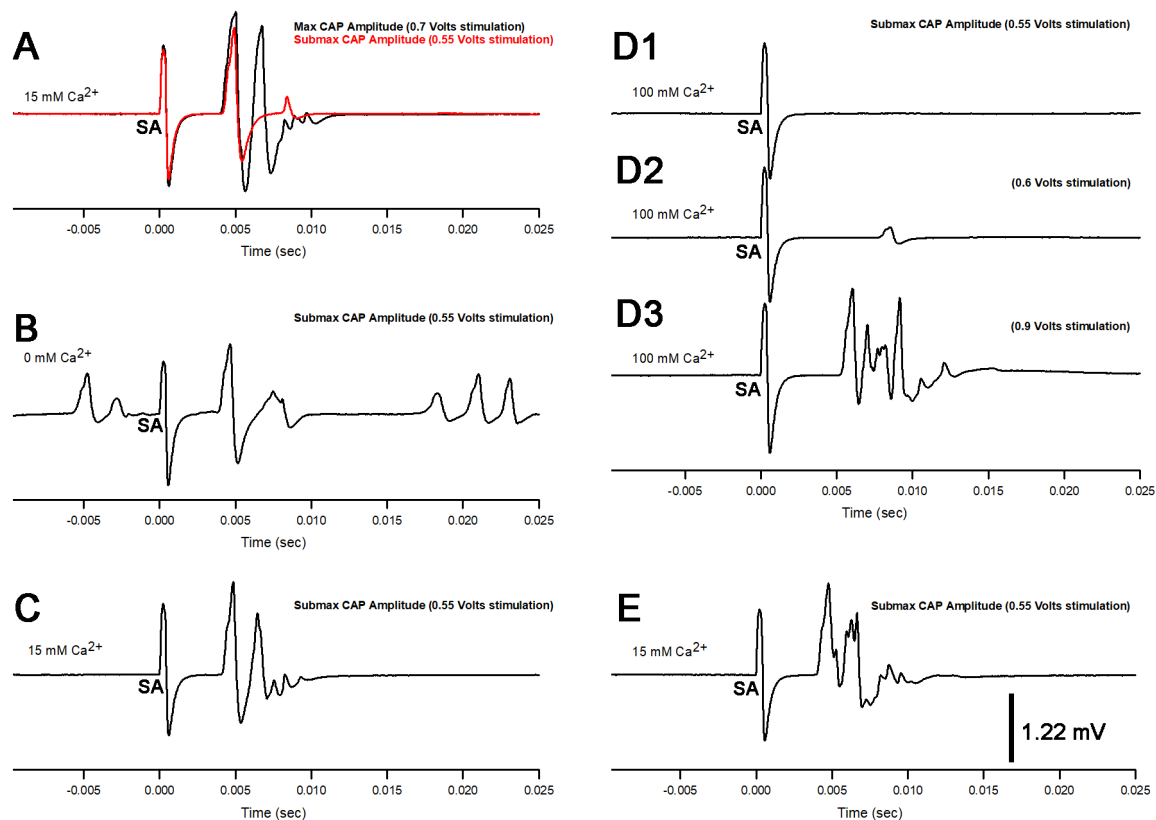line) in standard saline (**B**) With submaximal stimulation of the nerve produced random CAPs along with the evoked stimulation when exposed to saline without Ca^{2+}. (**C**) The random CAPs ceased when returning to standard saline, although the evoked CAP had recruited more neurons. (**D1**) With high [Ca^{2+}] exposure the amplitude of the CAP was reduced and even inhibited at a given stimulation voltage. However, the neurons were recruited with increased stimulus voltage (**D2,D3**). (**E**) Upon returning to standard saline and waiting 10 min the nerve was hyperexcitable as the CAPs are broad and complex.

3.2. Refractory Periods

The absolute and refractory periods are hard to assess in this PD nerve due to the complex shape of the CAPs. To ensure that more neurons are not recruited due to the increased excitability of the neurons due to low Ca^{2+} or that some drop out (or too small to detect) with high [Ca^{2+}]$_o$, the same set of neurons were fully recruited before and during the changed bathing media. We found a sub-maxim recruitment to obtain a simple shaped CAP was not feasible to reliably assess changes in refractory periods in many preparations as the CAPs changed in amplitude with a Ca^{2+} free bath. In some cases where the shape of the CAP was deemed to be a maximum amplitude and not changing in shape, where a particular peak of the secondary CAP was able to be followed through the initial complex CAP, the absolute refractory periods did not appear to show a consistent increase or decrease in timing for the Ca^{2+} free bath or for the 45 mM Ca^{2+} bath.

An example of changing the bathing Ca^{2+} from 15 mM to zero Ca^{2+} is shown in Figure 11. As illustrated an increase in the absolute refractory period occurred when reducing the [Ca^{2+}].
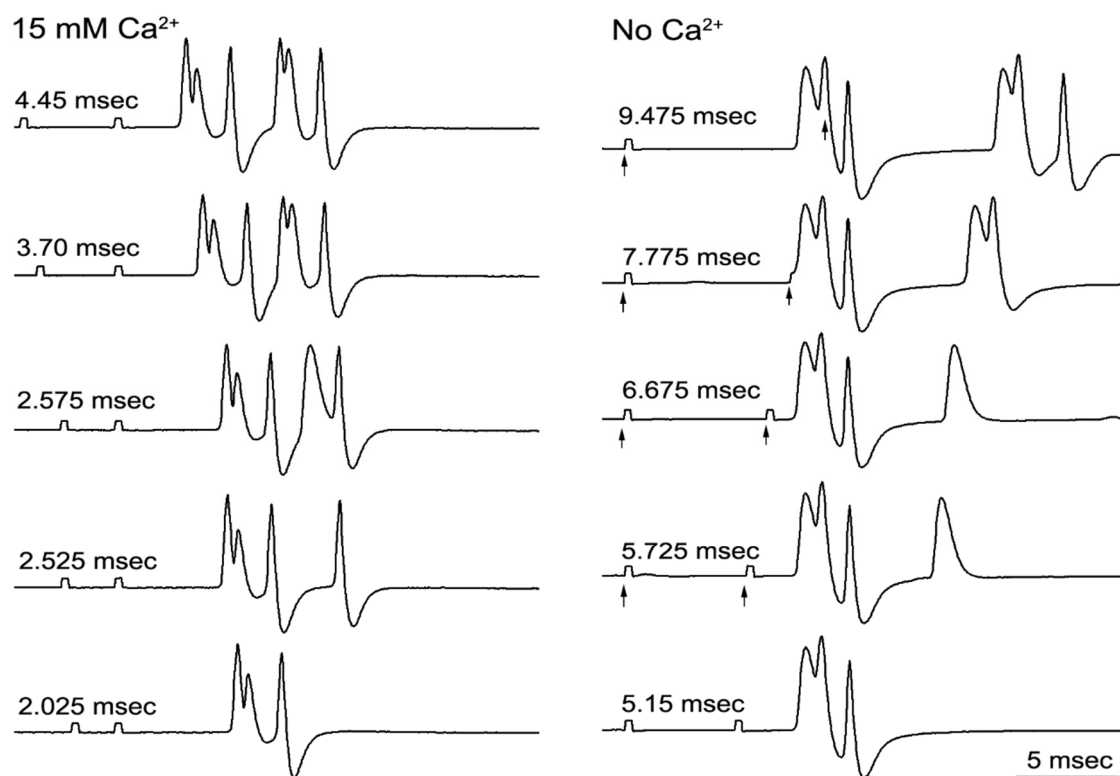
Figure 11. Exposure to reduced Ca^{2+} did not produce consistent changes to the absolute refractory period of the isolated PD nerve among preparations. In the example shown, the absolute refractory period increased in time with lower Ca^{2+}. The same nerve is used in this example with 15 mM (**left** column) and no Ca^{2+} (**right** column).

Given the variability noted and the difficulty to ensure a peak being followed is not overriding on a part of the initial complex CAP, it is difficult to state if refractory periods are shortening or elongating due to altering the extracellular Ca^{2+} concentrations. In addition, when changing the bath from 15 mM to no Ca^{2+} added, the spontaneous firing of the nerve made it difficult to obtain a constant shape of the CAP for most of the recordings due to overlapping random CAPs.

3.3. BaCl$_2$ Experiments

The potential action of $K_{(Ca)}$ channels in altering the PD organ activity or the electrical conduction along the PD nerve was examined by use of $BaCl_2$. $BaCl_2$ is known to depress $K_{(Ca)}$ currents due to Ba^{2+} going into the cell through voltage gated Ca^{2+} channels. The Ba^{2+} does not serve as a surrogate for Ca^{2+} in activating the $K_{(Ca)}$ channels. However, the assumption is that Ca^{2+} channels are present for Ba^{2+} to rise in concentration intracellularly close to the $K_{(Ca)}$ channels. The effect of adding Ba^{2+} to the standard saline and to have Ba^{2+} present when Ca^{2+} was absent provided some interesting results. With Ba^{2+} (15 mM) added to the standard saline no significant effects were noted to the activity profile when the joint was displaced (Figure 12A). Exposure to saline without Ca^{2+} added but Ba^{2+} added at the same concentration of 15 mM resulted in fairly stable recordings as standard saline (Figure 12A). Returning the bathing media back to standard saline and then exposure to saline without Ca^{2+} added presented with an increase in random activity. This was expected from previous studies as mentioned above (Figure 12A). There was a significant increase in the average number of spikes for the saline without Ca^{2+} added as compared to the initial saline exposure as well as the saline flush (Figure 12A; $p = 0.005$, Friedman repeated measures analysis of variance on ranks, normality test with Shapiro–Wilk, one way repeated measures analysis of variance and post analysis with an all pairwise multiple comparison procedures with a Bonferroni t-test, $N = 6$). The slightly higher averages in the

number of spikes for saline with Ba^{2+} and saline without Ca^{2+} but added Ba^{2+} is the reason they were not significantly different. To normalize the initial differences in the number of spikes among preparations a percent change from the initial saline was determined for each individual preparation. In this analysis, there was a significant difference from saline with Ba^{2+} added to saline without Ca^{2+} and the saline flush (Figure 12B; $p = 0.004$, normality test with Shapiro–Wilk, all pairwise multiple comparison procedures with a Tukey Test, $N = 6$). In examining a percent difference of saline exposure of Ba^{2+} (15 mM) and no Ca^{2+} added to saline without Ca^{2+} added. All 6 preparations showed an increase in activity (Figure 12B right side; $p < 0.05$, Wilcoxon rank sum non-parametric analysis).
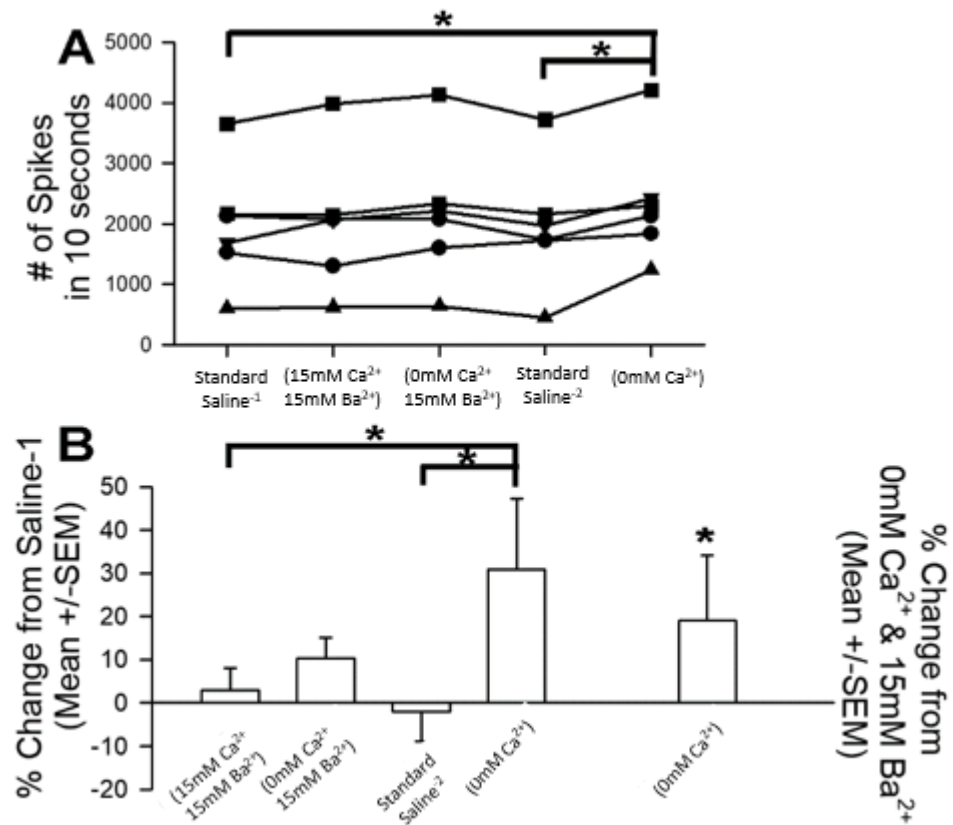

Figure 12. The effect of alterations in exposure to Ba^{2+} and/or Ca^{2+} on PD organ activity with PD joint displacement. (**A**) An experimental paradigm of changes the bathing saline consisted as standard saline, standard saline with 15 mM Ba^{2+} added, 15 mM Ba^{2+} and no Ca^{2+}, back to standard saline and lastly saline without Ca^{2+} added. Each line is an individual preparation and each symbol is an average of three trials of responses from the initial 10 s in activity or in the initial displacement. There is a statistical difference (*) between the initial saline to no Ca^{2+} exposure and from the second standard saline exposure to no Ca^{2+} exposure ($p = 0.005$, Friedman repeated measures analysis of variance on ranks, normality test with Shapiro–Wilk, one way repeated measures analysis of variance and post analysis with an all pairwise multiple comparison procedures with a Bonferroni *t*-test, $N = 6$). (**B**) To normalize the variability among preparations, a percent change from the initial saline was performed and statistical difference for the percent changes was shown for saline with added Ba^{2+} (15 mM) to the saline without Ca^{2+} added as well as the standard saline prior to saline without Ca^{2+} added ($p = 0.004$, normality test with Shapiro–Wilk, all pairwise multiple comparison procedures with a Tukey Test, $N = 6$). Comparing a percent change in saline with Ba^{2+} but no Ca^{2+} added to standard saline without Ca^{2+} added provided an increase in the percent change for all 6 preparations ($p < 0.05$, Wilcoxon rank sum non-parametric analysis) as shown on the right side. The Y -axis has the same values as other percent changes.

To address the effect of altered Ba^{2+} and Ca^{2+} concentration on nerve activity, the isolated nerve was used to induce CAPs. The addition of Ba^{2+} (15 mM) produced a decrease in the CAP amplitude (Figure 13B; 6 out of 6 preparations, $p < 0.05$, Wilcoxon rank sum non-parametric analysis) and with the following saline exposure of Ba^{2+} but without Ca^{2+} added, a decrease in the amplitude occurred compared to initial saline but also an increase in occurrence of CAPs at random times with some small CAPs (Figure 13C; 6 out of 6 preparations, $p < 0.05$, Wilcoxon rank sum non-parametric analysis) to large amplitude ones as shown in Figure 13E. Upon flushing the saline with standard saline and then changing the bath to saline without Ca^{2+} added rapidly resulted in the occurrence of multiple random CAPs (Figure 13E; 6 out of 6 preparations, $p < 0.05$, Wilcoxon rank sum non-parametric analysis).

An overview of the trends in the PD organ activity and effect on CAPs for the saline exchanges in the paradigm of altering Ba^{2+} and Ca^{2+} concentrations is illustrated in Table 1.

Table 1. Effects of exposure to Ba^{2+} and changes in $[Ca^{2+}]$.

Condition	PD Organ	CAPs
Standard saline	Normal	Normal
Saline + Ba^{2+} (15 mM)	Normal	↓ Amplitude
Saline + Ba^{2+} (15 mM) & No Ca^{2+}	slight ↑ Activity	↓ Amplitude ↑ Activity
Standard saline	Normal	Normal
Saline no Ca^{2+}	↑↑ Activity	↓ Amplitude ↑↑ Activity

General trends in the number of spikes in the PD nerve with joint displacement and changes in the compound action potential shape in the isolated PD nerve in different bathing conditions with $BaCl_2$ and $CaCl_2$. Comparisons are made to the initial standard saline exposure.

3.4. Model

A schematic model to summarize the findings of this study on electrical excitability of the axon and potential mechanisms are shown in Figure 14. The shape of the theoretical action potential is shown in the middle of the figure with the effects lower $[Ca^{2+}]$ to the left and higher $[Ca^{2+}]$ to right. The explanation of the effects on the shape of the action potential are outlined in the figure legend.
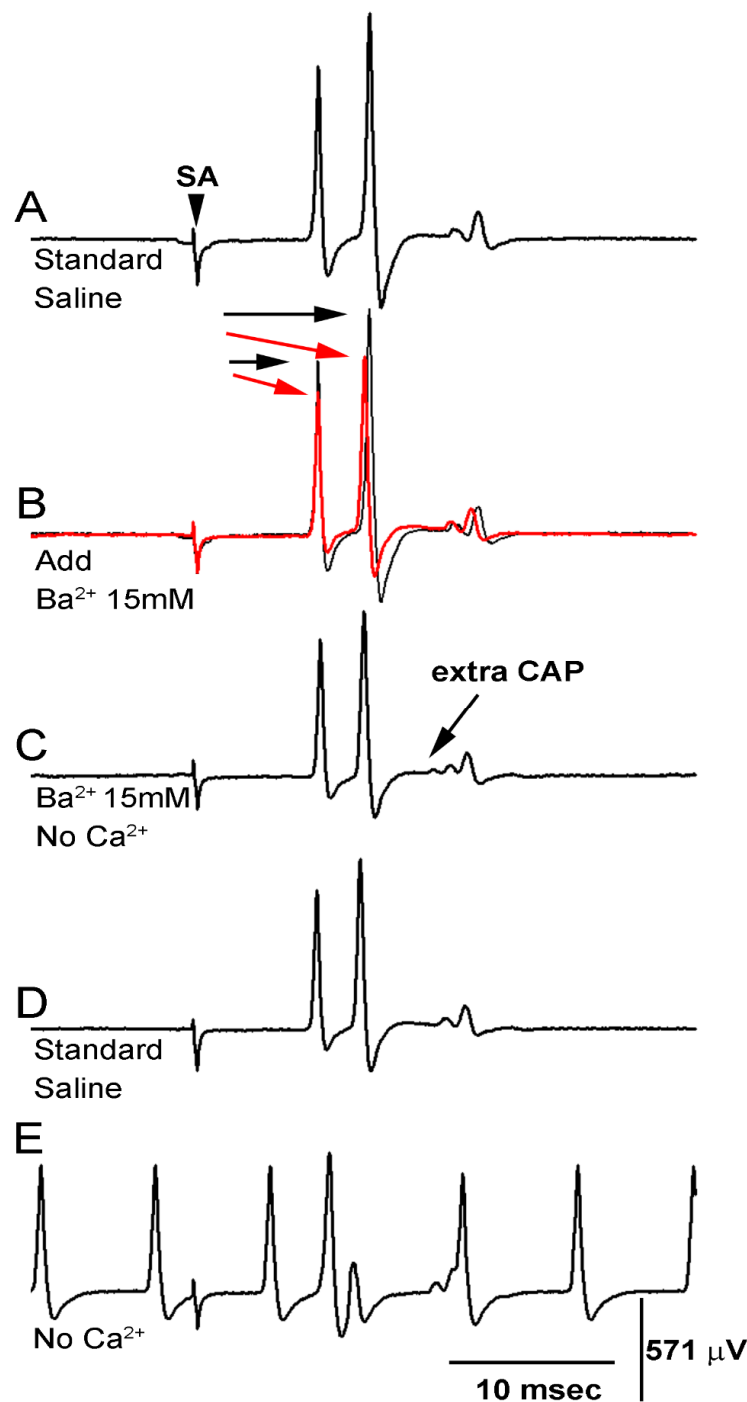
Figure 13. The effect of alterations in exposure to Ba^{2+} and/or Ca^{2+} on compound action potential (CAP) of the PD nerve. An experimental paradigm of changes is the same as shown in Figure 13 with the bathing saline consisted as (**A**) standard saline, (**B**) standard saline with 15 mM Ba^{2+} added, (**C**) 15 mM Ba^{2+} and no Ca^{2+}, (**D**) back to standard saline and (**E**) lastly saline without Ca^{2+} added. Representative single trials for each condition are shown. Note that exposure to Ba^{2+} decreases the amplitude of the CAP (**B**). The red trace is with 15 mM Ba^{2+} and the black trace is the same shown in A for standard saline. With 15 mM Ba^{2+} and no Ca^{2+} (**C**) a random CAP is shown riding on the normal evoked CAP. In the condition without Ca^{2+} in the saline (**E**) many random CAPs occur. The Y and X axis are at the same scales for all traces.

E_{Na} & E_K are the equilibrium potentials
Th is threshold
$[Ca^{2+}]_O$ is the Ca^{2+} concentration outside

1. Reduced threshold due to less Na_V inactivation by Ca^{2+}.
2. Increased amplitude due to less Na_V inactivation by Ca^{2+}.
3. Could be wider AP due to reduced $K_{(Ca)}$ activation.
4. Could be not as prolonged AP due to reduced $K_{(Ca)}$ activation.

1. Raised threshold due to more Na_V inactivation by Ca^{2+}.
2. Decreased amplitude due to more Na_V inactivation by Ca^{2+}.
3. Could be narrower AP due to increased $K_{(Ca)}$ activation.
4. Could be prolonged AP & hyperpolarization due to more $K_{(Ca)}$.
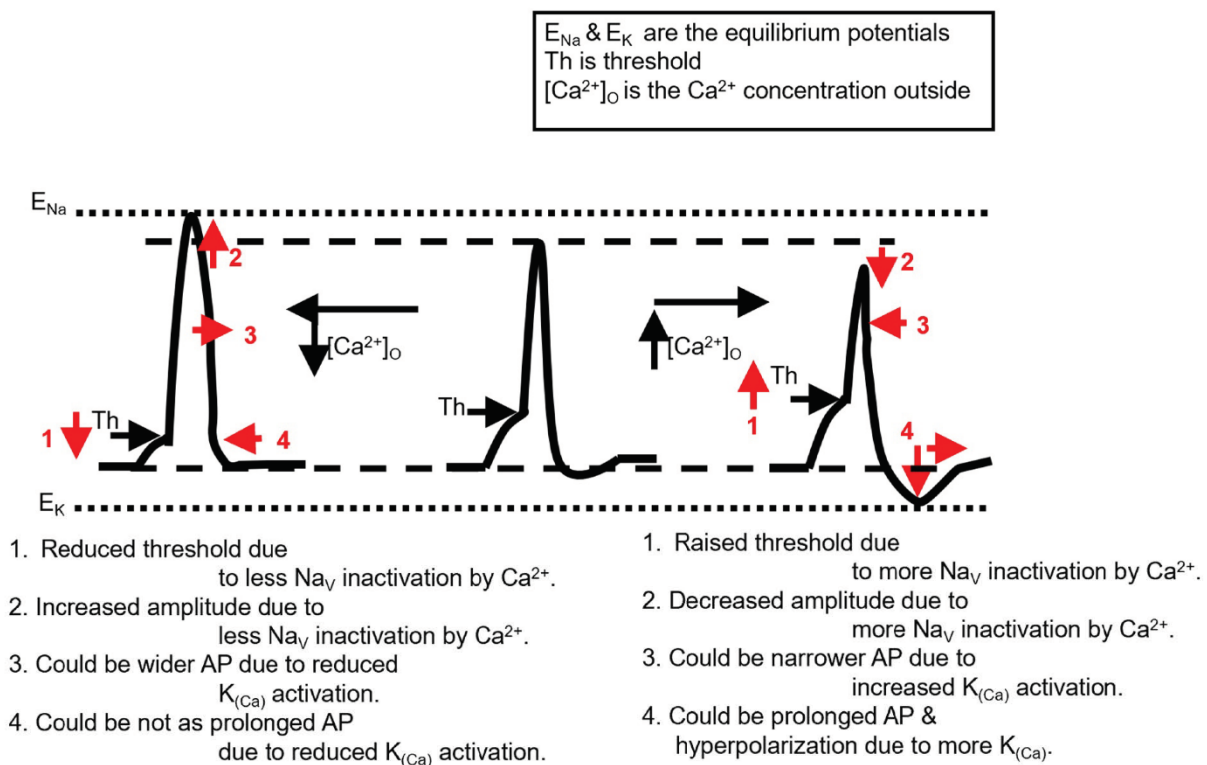
Figure 14. Theoretical model in how action potential amplitude and shape is altered by changes in $[Ca^{2+}]_o$.

4. Discussion

This study has substantiated that a higher $[Ca^{2+}]$ and $[Ba^{2+}]$ likely block the Na_V channels which leads to a decreased amplitude of CAPs and threshold of recruiting CAPs. Low $[Ca^{2+}]$ likely removes a Ca^{2+} block of Na_V channels resulting in lower threshold of activation resulting in prolonged activity of the neurons. In addition, this study aided in better physiological classification of the SAC in the crustacean chordotonal organs. Since activity was still present with reduced $[Ca^{2+}]$ it would imply the SACs prefer Na^+ ions and is a different SAC subtype than a PIEZO 2 and that these SACs are blocked by Mn^{2+} and not Mn^{2+} permeant. It was not clear, in these studies, if altering $[Ca^{2+}]$ and $[Ba^{2+}]$ had an impact on potentially present $K_{(Ca)}$ channels in the sensory endings or on the axons. It is not yet known if $K_{(Ca)}$ channels have a role in the excitability of these neurons.

It appears feasible that Ca^{2+} ions blocked voltage-gated ion channels and possibly influenced the kinetics of the various channels as Armstrong and Cota [9] noted to occur for GH3 (Growth hormone 3) cells from a rat pituitary cell line. Ca^{2+} may alter the screening of charges on membranes to account for the changes observed [20]; however, with the recording procedures used in this study this possibility was not able to be addressed. In conditions with saline devoid of the standard 15 mM of Ca^{2+} and containing 15 mM Ba^{2+}, the neurons were not highly hyperactive indicating that Ba^{2+} can substitute for Ca^{2+} in some manner preventing the threshold from large changes. In addition, like for Ca^{2+}, Ba^{2+} depresses the amplitude of the CAPs hinting that Ba^{2+} is binding or screening the Na_V channels in a similar manner. Surprisingly, Ba^{2+} did not decrease the PD organ activity while Mn^{2+} substantially blocked activity without noticeable changes on the CAPs of the isolated PD nerve. In an early study, 15 mM exposure for either the $MnSO_4$ or $MnCl_2$ did not show significant differences in the number of spikes for the PD organ, but 30 mM $MnSO_4$ did depressed activity [31]. $MnSO_4$ was the ionic form of Mn^{2+} also used in the study herein.

The SACs within the sensory endings of the crustacean chordotonal organs are not likely a PIZEO 2 subtype since this subtype is a Ca^{2+} permeant type in mammals [23]. Likewise, earlier pharmacology studies do not allude to these crustacean SACs as a PIEZO1

or DEG/ENaCs subtype [24–26]. The genomic or protein sequence for these SACs in crustaceans remains to be determined. The large nature of the cell bodies and easily isolation of the cell bodies, with identified function, open a ripe opportunity to molecular identity the SACs at a cellular level. Such identification would be beneficial for examining SACs in chordotonal organs in other crustaceans and arthropods. These SACs may be a unique subtype given the pharmacology and physiological profiles known.

The CAPs obtained in the PD were complex in waveform which made it difficult to establish refractory periods in the subgroups of the CAPs with changes in $[Ca^{2+}]$. In the few preparations where defined peaks in the CAPs were able to be identified while decreasing the interpulse interval between stimuli, the absolute refractory period did not show a consistent trend with changes in $[Ca^{2+}]$. Thus, it was not feasible to determine if there were alterations in the refractory periods. It would be beneficial to determine the absolute refractory periods with intracellular recording from single neurons to investigate if $K_{(Ca)}$ has an effect. Since low $[Ca^{2+}]$ produces random CAPs, it is hard to distinguish the evoked CAPs and a high $[Ca^{2+}]$ can dampen the amplitudes where they can be lost in the baseline.

Using the crustacean model to address the effects of altered $[Ca^{2+}]$ on neuronal excitability is useful for a fundamental understanding on the mechanisms altering excitability. However, there are substantial differences as compared to mammalian neurons in that these crustaceans are marine organisms with higher ionic concentration in the cytoplasm as well as in the extracellular fluid and saline to mimic the ionic makeup of extracellular fluid. In addition, the PD neurons are unmyelinated, but do have a connective sheath which holds the neurons in a separated nerve bundle from the main leg nerve. Understanding the evolutionary differences in managing electrical excitability and conduction in neurons among animals can help in better addressing what fundamental properties are shared among organisms [32].

To directly address if $K_{(Ca)}$ channels are present in the PD nerve and sensory endings it would be useful to be able to obtain intracellular recordings from the cell body. In examining a longer exposure of extracellular Ba^{2+} substitution for Ca^{2+} or examine potential pharmacological agents known to impact $K_{(Ca)}$ channels while recording evoked action potentials would allow one to establish any changes in the shape of the action potentials and if any after hyperpolarization was impacted. In addition, determining if refractory periods are altered with changes $[Ca^{2+}]$ in would be able to be obtained. The attempts to date to obtain intracellular recordings have not been fruitful due to rolling of the soma and the connective tissue around the soma have made it difficult. In the past, loose-patch recording has been able to be made on somas of the neurons for chordotonal organs in crabs to identify neuronal function with movements of the chordotonal strand [33]. This technique was only feasible for the larger somas for the dynamic sensitive neurons but not the small somas for the static position sensitive neurons. It might be feasible to use a loose-patch electrode to hold the cell body in place while impaling the cell with a sharp glass microelectrode. In addition, a loose patch recording over the scolopedium, which contains the sensory endings, might be feasible to record the currents of the SACs to determine if they are solely Na^+ driven currents.

The reproducibility in physiological recordings and data analysis of previously recorded data sets from participants within a neurophysiology course revealed the same trends as reported herein. Sixteen participants in groups of two conducted the same set of experiments in altering the concentration of Ca^{2+} and effects of Ba^{2+}. The classroom setting did not have vibration free tables and each group might have moved the joint at various rates; however, the same general trends occurred. In addition, data collected earlier in a research laboratory, as presented herein, analyzed 3 different data sets (one of no Ca^{2+}, 45 mM Ca^{2+} and the paradigm with changes in Ba^{2+} and Ca^{2+} in the saline). All analysis showed the same trend in increasing or decreasing the number of spikes; however, the absolute values differed. Such differences occur due to the observer choosing a different threshold from the baseline in detecting a spike. In using standardized software for analysis such as Chart

7 or Chart 8 (ADI Instruments, Colorado Springs, CO, USA) for detecting a deflection from baseline to count a spike was kept consistent throughout the analysis within a data set but varied depending on the group analyzing a data set. This is an important issue to mention as raw and/or compiled data are generally made available with published reports. Depending on what one may choose as a threshold for analysis in determining a response a different absolute value in the number of spikes will be obtained. As highlighted in an earlier report on similar data analysis [28], this topic is an issue when implementing citizen science or crowdsourcing projects to analyze data sets [34] as well as providing open source data without extensive explanation for every data set. This can be a large undertaking to annotate each data set to reproduce the exact raw counts, while the trends will be similar despite some variation choosing a threshold for analysis.

This ACURE (authentic course-based undergraduate research experiences) [27,30,31,35] approach we have taken with this project builds on the CURE (course-based undergraduate research experiences) concept [36,37]. This provides an exposure to a research experience as a team within a course setting.

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