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Amiloride hydrochloride does not have a significant effect on crayfish muscle extensor cells pH_i

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ABSTRACT

Amiloride is a diuretic drug known for its ability to regulate K^+ levels and prevent the body from absorbing excessive levels of Na^+ by inhibiting FMRF-amide gated Na^+ channels (FaNaC channels). Amiloride has the ability to reduce the effect of FMRF-amide peptides on the synapses in the crayfish neuromuscular junction. However, in addition to its ability to block FaNaC channels, it is possible that Amiloride may have offset effects that inhibit normal functioning within the cell. We therefore hypothesize that the addition of Amiloride will make the intracellular pH more acidic. We used fluorescence microscopy to quantify changes in intracellular pH in crayfish extensor muscle cells by comparing the changes in fluorescence between a Ringer's solution and a solution containing Ringer's and Amiloride. Through doing this, we found that while the data was not statistically significant, it did tentatively support our hypothesis.

INTRODUCTION

Voltage-gated ion channels play a significant role in synaptic transmission at the neuromuscular junction. Voltage-gated sodium channels in particular regulate the influx of Na^+ in response to membrane potential changes. This regulation of the amount of Na^+ ions inside and outside of the cell directly affects synaptic transmission through the normal depolarization of the cell to fire an action potential. A lower presence of Na^+ inside the cell lowers the amount of neurotransmitters released, leading to weaker excitatory postsynaptic potential measurements (EPSPs).

Amiloride prevents excessive influx of Na^+ by inhibiting FaNaC channels (Schaffhauser et Al 2016). FaNaC belongs to the larger ion channel family Deg/ENaC. ENaC allows Na^+ to enter the cell and contributes to ion transport across epithelial barriers. FaNaCs can be activated by external protons or the cell's pH level. Amiloride is known to block ENaCs and DEGs; this is because when amiloride is in an environment with a high pH, it breaks apart and neutralizes the pH levels in the environment. Because FaNaCs react to the pH levels, Amiloride reduces their activity.

Amiloride is known to inhibit plasma membrane-bound transport systems by binding to sodium channels in cellular tubules to block sodium reabsorption. Amiloride acts presynaptically to inhibit an induction of neurotransmitter release; however, it does not prohibit action potential propagation in nerve terminals (Smith 2000). Although Amiloride is known to prohibit normal functioning of FaNaC voltage gated sodium channels, it is possible that an additional offset reaction can inhibit normal functioning of the Na^+/H^+ exchanger, the H^+ pump, or the HCO_3/Na^+ exchanger, leading to a change in pH within the cell (pH_i). Further research is necessary to better understand the linkage between Amiloride and its effects on intracellular function.

We predict that by blocking the normal functioning of the voltage gated FaNaC sodium channels, Amiloride will have an offset effect of additionally interfering with the active extrusion of H^+ from cells. We hypothesize that Amiloride will lead to a buildup of H^+ ions within the cell, thus decreasing pH_i. Because intracellular pH is a vital aspect of all cellular processes (i.e. membrane potential, transportation across the cellular membrane, and ability of muscle cells to contract), we hope to better understand the secondary effects of Amiloride in invertebrates by specifically examining the role it may play in maintaining pH_i. We ultimately found that intracellular pH decreased with the addition of Amiloride although this effect was not statistically significant.

MATERIALS AND METHODS

Crayfish Dissection

Crayfish specimens were first anesthetized in an ice bath for 15 minutes, and then dissected according to a procedure described by Atwood and Parnas (1968). Following this procedure, we used scissors to remove the organism's abdomen and make cuts along the edge of the ventral side of the crayfish. We removed the ventral section of the exoskeleton and the swimmerets and scraped off the flexor muscles and digestive tract with a thumb, leaving the dorsal

extensor muscles attached to the exoskeleton. These are the muscles that we examined for changes in fluorescence. We used a new crayfish for each day of experimentation.

Solutions

We used a normal Ringer's solution composed of 5.4 mM KCl, 196 mM NaCl, 2.6 mM MgC•6H2O, 10mM Hepes Buffer, and 13.5 mM CaCl2•2H2O. For the positive control solution, we replaced the 196 mM NaCl with 196 mM Propionic acid. We also used 300 µM Amiloride which was added to Ringer's.

pH_i Measurement

To test our hypothesis that the addition of Amiloride makes the intracellular pH more acidic, we completed a series of tests. We used BCECF-AM and fluorescence microscopy to measure the changes in fluorescence (which correspond to changes in pH_i) in crayfish extensor muscle cells. To do this we followed the procedure described by Lindgren, Emery, and Haydon (1997). BCECF-AM is designed to act hydrophobically so that it can move across the phospholipid bilayer into the cell. Esther groups then cut off the AM group, trapping the BCECF dye in the cell.

The BCECF-AM was put in Ringer's solution and allowed to set for one hour, then it was rinsed with ringers and allowed to sit for another hour. We then took 10 images of the crayfish extensor muscle cells in the Ringer's. Then an Amiloride (or Propionic Acid) and Ringer's solution was put on the crayfish and allowed to sit for 20 minutes before we took 10 more images. We finished the process with a wash of normal Ringer's and waited another 20 mins before taking the final 10 images. This was repeated for each crayfish used.

We used a Nikon Eclipse 50i fluorescence microscope and collected images using Nikon Elements software and a Nikon DS-Ri1 camera. The wavelength emitted by BCECF-AM was greater than 515nm and the wavelength of the light used to excite it was less than 488nm. We ran 3 positive control experiments with 196 mM Propionic acid to show that the method using BCECF-AM could detect a change in pH. Propionic acid is a relatively strong acid with a pH of 2.94 that has few secondary effects on the cell. We used fluorescence microscopy and image processing to measure quantifiable changes in pH_i from the measured changes in fluorescence. We then used the same process mentioned above to run a series of tests using both BCECF-AM and Amiloride and observed any changes in fluorescence/pH_i.

Changes in pH_i were quantified from our images using ImageJ (NIH). While taking our measurements and images, we recorded all changes in fluorescence emission intensity in Excel by calculating $\mathbf{\Delta}F/F_R$ (the change in fluorescence emission intensity from the control that correspond to changes in pH_i). We also took the average fluorescence measurements from all solutions and ran t-tests to determine if the changes we observed were significant.

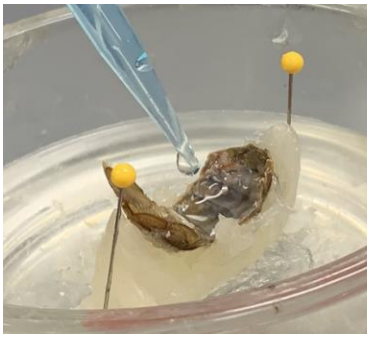

Figure 1. Crayfish boat made of wax and vaseline used to hold BCECF-AM solution.

Because BCECF-AM is an expensive chemical, we made a boat out of wax and Vaseline to hold the crayfish tail and keep the solution sealed inside. This allowed us to fit 1 ml of the solution inside the crayfish tail so that there was limited waste (Fig. 1). This posed a possible difficulty at the start, but through trial and error the boat proved to be an adequate solution and allowed us to carry out the experiment in an effective manner.

RESULTS

To analyze the effect of Amiloride on pH_i, we first loaded the crayfish muscle extensor cells with the pH indicator BCECF-AM. We then measured the fluorescence emission of the cells while also suspended in Propionic acid. The positive control Propionic acid showed high levels of fluorescence in comparison to normal Ringers, with similarly high levels seen after a wash step (Fig.2).

A fluorescence test quantified the difference in fluorescence due to changes in pH_i between crayfish muscle cells exposed to normal Ringer's solution and Amiloride. We calculated fluorescence levels by comparing the variable solution mean fluorescence levels to normal Ringers F/F_R.

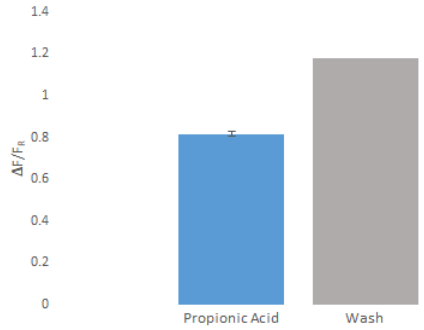

Figure 2. The average F/F_R of $[pH]_i$ of Propionic acid and wash. Error bars indicate the standard error and range of values of the recordings taken for each solution. Propionic Acid: n=3 Wash: n=1

Figure 3 shows the results of an individual experiment where Amiloride was shown to lower fluorescence, then after a wash step fluorescence was shown to increase again. This was consistent with our other experiments (Fig. 4), with the exception of our first trial.

In the first experiment, the fluorescence increased, which is consistent with an increase in intracellular pH. In the remaining five experiments, fluorescence was shown to decrease. A typical example of this trend is shown in Figure 3. Thus, the data plotted represents the latter five data points. This was done to correct and account for any error due to a lack of technique or experience with the fluorescence microscopy method.

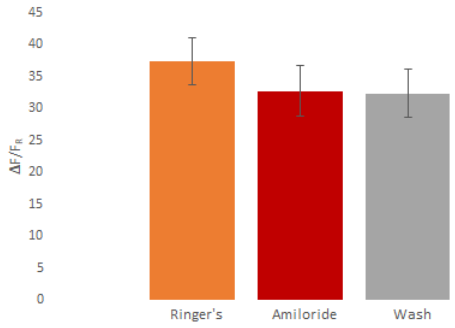

Figure 3. The fluorescence of crayfish extensor muscle cells with Ringer's, Amiloride, and wash solutions. Error bars indicate the standard error and range of values of the recordings taken for each solution. n=1

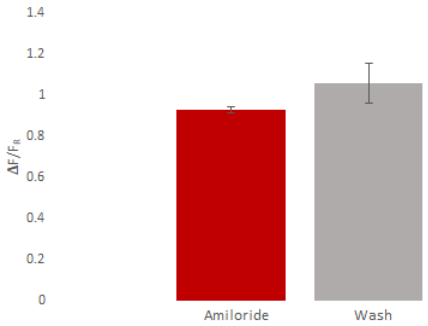

Figure 4. The average F/F_R of Amiloride and wash. Error bars indicate the standard error and range of values of the recordings taken for each solution. n=5

DISCUSSION

To test the effects of Amiloride on intracellular pH, we used fluorescence microscopy to measure quantified changes between Ringer's and Amiloride solutions. As a positive control, we used Propionic acid to demonstrate that the methods of measuring fluorescence were effective. Before testing amiloride we wanted to test our ability to measure pH with Propionate. After proving BCECF's effectiveness with Propionate (Fig.2) we continued our experimentation by testing Amiloride. We conducted five experiments where we applied amiloride; the first experiment showed an increase in overall fluorescence while the remaining four showed an overall decrease. A typical example of the latter four experiments is shown in Fig.3.

Our positive control experiment was successful, but we found that the wash did not return to fluorescence emission levels similar to those in the Ringer's solution. This is presumably because there was not a strong enough gradient for the Propionic acid to diffuse out of the cell. This experiment demonstrated that Propionic acid diffuses into the cell and dissociates to H^+ and propionate, leading to its inability to be simply washed out of the cell. The gradient driving the hydrogen propionate into the cell diffuses very slowly and the wash step starts a reversal; however, there is not a large enough gradient to make a difference. Therefore, the BCECF-AM indicator detects a drop in pH yet cannot be fully washed out.

Although our other data was not statistically significant, our experiment supported the hypothesis that pH_i would become more acidic with the addition of Amiloride.

While our first set of data showed the pH_i becoming less acidic, the remaining five tests became more acidic with the addition of Amiloride. This is why we disregarded the first set of data in our final

data analysis. By repeating these tests, and therefore gaining more data points, the results could become statistically significant, and might further affirm that the first test was an outlier in the data set.

In most cells pH_i is maintained at around 7.0, making it relatively acidic compared to the extracellular fluid. Free-floating H^+ ions can increase the acidity of their surrounding solution. When these H^+ ions enter into the cell the pH_i becomes increasingly acidic. Oftentimes changes in pH_i are in response to externally applied inhibitory/regulatory substances, like the Amiloride used in the experiment. Thus, cells must regulate pH_i with Na^+/H^+ exchangers, H^+ pumps, and HCO_3 /Na^+ exchangers (Putnam 2016). The Na^+/H^+ exchanger is a membrane protein that facilitates movement of Na^+ into the cell and H^+ out of the cell. This is important to the functioning of the cell because changes in pH can alter the chemical structures of proteins and thus their function and overall functionality of the cell. To regulate pH_i, there is an H^+ pump that solely moves H^+ out of the cell through the use of ATP (Demaurex et al., 1997). Both the Na^+/H^+ exchanger and the H^+ pump are responsible for raising the pH_i, but the HCO_3 /Na^+ exchanger lowers the $[pH]_i$ (Wang et al., 2000). The blockage of any of the first two systems and/or activation of the third could be responsible for the change in pH_i that we saw in the crayfish extensor muscle cells.

Although we measured pH_i in muscle cells, these changes would also likely be occurring in other places, such as nerve terminals. Further experimentation is needed to see which of the aforementioned pumps could be the cause for the measured changes in pH_i. A separate experiment could include individually blocking each of the systems that regulate pH_i to see which results are consistent with those of Amiloride.

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REFERENCES

Demaurex, N., Romanek, R.R., Orlowski, J., & Grinstein, S. (1997). ATP Dependence of Na+/H^+ Exchange - Nucleotide Specificity and Assessment of the Role of Phospholipids. J Gen Physiol, 109(2): 117-128.

Atwood, H.L., Johnston,H.S. (1968). Neuromuscular synapses of a crab motor axon. The Journal of Experimental Zoology., 167(4), 457–470.

Lindgren, C A, Emery, D G, Haydon, P G, and Lindgren, C A. (1997). Intracellular Acidification Reversibly Reduces Endocytosis at the Neuromuscular Junction. The Journal of neuroscience : the official journal of the Society for Neuroscience 17, no. 9, 3074–3084.

Robert W. Putnam, (2016) Chapter 17 - Intracellular pH Regulation, Nicholas Sperelakis, Cell Physiology Source Book (Fourth Edition), Academic Press, 2012, Pages 303-321.

Santos-Torres, J., Ślimak, M. A., Auer, S., & Ibañez-Tallon, I. (2011). Cross-reactivity of acid-sensing ion channel and Na+–H^+ exchanger antagonists with nicotinic acetylcholine receptors. The Journal of physiology, 589(21), 5109-5123.

Schaffhauser, D., Fine, M., Tabata, M., Goda, T., and Miyahara, Y. (2016). Measurement of Rapid Amiloride-Dependent pH Changes at the Cell Surface Using a Proton-Sensitive Field-Effect Transistor. Biosensors (Basel), 6(2):11.

Smith, A. B., Motin, L., Lavidis, N. A., & Adams, D. J. (2000). Calcium channels controlling acetylcholine release from preganglionic nerve terminals in rat autonomic ganglia. Neuroscience, 95(4):1121-7.

Wang, C.Z., Yano, H., Nagashima, K., and Seino, S. (2000). The Na+-driven Cl−/HCO3− Exchanger cloning, tissue distribution, and functional characterization. Journal of Biological Chemistry, 275, 35486-35490