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Amiloride Inhibits Synaptic Transmission at Crayfish Neuromuscular Junctions, but no Evidence Suggests Its Interaction with DRNFLRFamide.

CAMILA HASSLER, JOSIE NOLAND, and SOPHIE SMITH
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

FMRFamide-activated sodium channels (FaNaCs) are similar to mammalian acid-sensing ion channels (ASICs). We analyzed the reaction of FaNaCs exposed to DRNFLRFamide (DF2) at the crayfish neuromuscular junction. We measured excitatory postsynaptic potentials (EPSPs) after exposing crayfish extensor muscle cells first to DF2, then DF2 and amiloride, and finally, amiloride alone. Contrary to previous studies, DF2 inhibited synaptic transmission. Amiloride also inhibited synaptic transmission. Therefore, FaNaCs are similar to ASICs in regard to their response to amiloride. The results were inclusive in determining the interaction between amiloride and DF2.

INTRODUCTION

FMRFamide-activated sodium channels (FaNaCs) are sodium-selective ion channels found in invertebrates. In order for a FaNaC to open, a natural or synthetic FMRFamide must be present. FaNaCs respond quickly to the presence of a FMRFamide. We used crayfish as our model organism because they are inexpensive and have nerves that are easy to find under a microscope.

DRNFLRFamide (DF2) is a peptide that activates FaNaC channels (Cottrell, 2005). DF2 prompts depolarization, along with decreased input resistance. It also increases Ca^{2+} current, which then activates muscle contraction in crustacean ventral muscles. The increased Ca^{2+} current results in an increase of neurotransmitter release from the presynaptic cell (Weiss, 2003). Amiloride is a small molecule diuretic, that has been used to dissect sodium transport pathways in many different systems. It interacts with the epithelial sodium channel and acid-sensing ion channel proteins. In the medical field, amiloride is frequently used to treat high blood pressure.

DF2 is a known activator of FaNaCs, and amiloride is known to block the effects of DF2 in mammalian acid-sensing ion channels (ASICs). We wanted to better understand the relationship between ASICs and FaNaCs—especially if amiloride has the same inhibitory effects of FaNaCs in both channels. We tested if amiloride inhibits the effect of the FMRFamide DF2 on crayfish neuromuscular junctions. We selected this question to explore FaNaCs and their correlation to excitatory postsynaptic potentials (EPSPs). FaNaC channels are similar to ASIC channels, so we hypothesized amiloride would inhibit the FaNaC that becomes activated by DF2.

Our study investigated the following:

1. Are FaNaCs similar to ASICs in how they react to amiloride? Will EPSP amplitude be reduced after crayfish extensor muscles have been exposed to amiloride?
2. How significant of a change in EPSP amplitude will occur when DF2 and amiloride are used in combination?

We expected DF2 alone would increase EPSPs, as it is a known activator of FaNaCs. However, amiloride alone should not impact the EPSPs at all, because the FaNaCs have not yet been activated (unless there are naturally occurring FMFRamides in the crayfish). We anticipated the trial in which the crayfish is exposed to DF2, followed by amiloride would show the inhibitory effects of amiloride.

Our results show amiloride depressed synaptic potential at crayfish neuromuscular junctions. Surprisingly, DF2 also depressed synaptic potential at crayfish neuromuscular junctions. Lastly, DF2 combined with amiloride had a large margin of error, so conclusions cannot be drawn. However, the large range of EPSP values suggest there was interaction between the chemicals.

MATERIALS AND METHODS

Dissection and Preparation

To prepare for dissection, we anesthetized a crayfish with an ice bath. We began the dissection by removing the tail using scissors. Then, we made two lateral cuts on each side of the tail and removed the exoskeleton on the ventral side to reveal the underlying muscle. To reveal the desired dorsal extensor muscles, we carefully used our

thumbs to scrape off the interior crayfish muscles and fat stores—being sure to remove all of the gastrointestinal tract to prevent contamination. Once the dissection was complete, we placed the specimen in 100mL of crayfish Ringer's solution. After completing a control reading, we also added DF2 and/or amiloride to the solution depending on the particular trial being completed.

Condition	Average EPSP
Control	25.3 mV
Amiloride	2.4 mV
DF2	13.7 mV
Both	11.4 mV

Table 1. This table shows the average EPSP for each condition. Percent change values were comparisons between average EPSP of the control and other conditions.

Preparing Micro-electrodes

We created two different types of electrodes using glass capillary tubes and a micro-electrode puller. We first made a micro-electrode with which we measured intra-cellular EPSPs. We filled the electrode with three molar potassium chloride (KCl). The measuring electrode cannot contain any bubbles (otherwise the resistance would be off), so we inverted the electrode and used light tapping to remove any potential bubbles. Then we placed the electrode on a micro-manipulator that allowed us precision entering cells and protection from large, uncontrolled movements that would likely break the electrode tip.

We also created suction electrodes which were used to gather and stimulate individual neurons. First, we pulled a standard electrode on the micro-electrode puller. Then, we gently filed the tip to produce an opening large enough to admit a nerve axon.

Intracellular Recording and Electrical Stimulation

We first measured EPSPs of crayfish extensor muscles exposed to only normal Ringer's solution (5.4 mM KCl, 196 mM NaCl, 2.6 mM MgCl2 x 6H2O, 10 mM HEPES, and 13.5 mM CaCl2 x 2H2O). The suction electrode stimulated a neuron by applying a supra-threshold stimulus, while the micro-electrode measured EPSPs in corresponding muscle cells. Stimulation was applied using a Grass SD9 Stimulator. We used LabChart programming to record our data.

RESULTS

We explored how amiloride and DF2 impacted synaptic transmission at crayfish neuromuscular junctions. We ran four trials in which we recorded EPSPs. The control trial featured physiological crayfish Ringer's solution. Then, we added 300 micro-molar amiloride to the 100mL Ringer's solution. After five minutes, we recorded EPSPs for the amiloride trial. Then, we disposed of the amiloride solution and filled the dish with 100mL Ringer's solution and fifty nano-molar DF2. We repeated the process of recording EPSPs after DF2 exposure. Our last trial combined the DF2 solution with 300 micro-molar amiloride. Then we recorded EPSPs after exposure to both chemicals simultaneously. The average EPSP amplitude for each condition is shown in Table 1.

We hypothesized adding amiloride and DF2 would result in a decrease in EPSP amplitude. Our raw data supported our hypothesis because the EPSP amplitude of the trial with amiloride and DF2 was 11.4 mV, 13.9 mV less than the control. However, we also predicted the application of DF2 would increase EPSP amplitude because it is a known activator of ASICs. Our results reject our hypothesis regarding DF2 exposure increasing

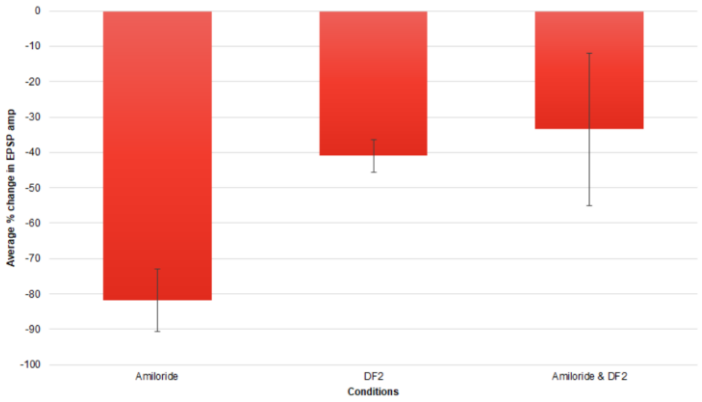

Figure 1. This figure shows the average percent change in EPSP amplitude of the variable conditions compared to the control. The average percent change in EPSP amplitude for only amiloride was -81.8%. The average percent change in EPSP amplitude for only DF2 was -41.0%. The average percent change in EPSP amplitude for both DF2 and amiloride was -33.5%.

FaNaC activation. We found DF2 had the opposite—lowering EPSPs.

Condition	P-Value
Both	0.787
Amiloride	0.068
DF2	0.072

Table 2. This table shows the p-values for three conditions.

According to our p-values as shown in Table 2, our results were inconclusive in finding significant changes in synaptic transmission after exposure to amiloride or DF2 at crayfish neuromuscular junctions. However, we cannot ignore the difference of average EPSP (shown in Figure 1) between amiloride alone versus amiloride and DF2. We collected marginally significant data suggesting a relationship between amiloride and DF2. However, our large margin of error makes our results more difficult to analyze.

DISCUSSION

We expected DF2 alone would increase EPSPs and amiloride alone would have no effect. We anticipated a trial with both DF2 and amiloride together would be the only way to witness the inhibitory effects of amiloride. We expected these results because DF2 is a known activator, amiloride is an inhibitor, and FaNaCs must be open for amiloride to demonstrate inhibitory effects.

The data we collected is inconclusive in determining the interaction between amiloride and DF2. Our margin of error is too large to assign significant value to our data. While Figure 1 shows a slight difference between DF2 alone and DF2 with amiloride, our statistical evaluation of the conditions only shows marginal significance.

However, amiloride alone created a large depression in synaptic transmission. This was expected because amiloride is a known inhibitor of ASICs, and we expected FaNaCs to respond similarly. Our results with DF2 were surprising—having the opposite effect than we expected. Previous research has shown DF2 activates crayfish synapses, but our data shows DF2 inhibiting them. While our results prove inconclusive in determining a relationship between DF2 and amiloride, it is possible DF2 prevented an even larger inhibition of synapses. The large margin of error associated with our data could signify an interaction between DF2 and amiloride over time. Regardless, the data is minimally helpful in forming conclusions.

In another study, DF2 was tested in crayfish with several enhancers and inhibitors. The inhibitors used in this experiment were Rp-cAMPS (which inhibits PKA), and Rp-8-pCPT-cGMPS (which inhibits PKG). The inhibitors alone barely altered EPSPs and barely inhibited the response to DF2. However, used together, the two inhibitors totally blocked the effect of DF2. (Badhwar, 2006)

We found a similar result in our experiment. DF2 combined with amiloride did not inhibit FaNaCs. We were interested in exploring FaNaCs because of their relevancy to understanding ASICs. Both FaNaCs and ASICs contain fundamental functions to pathological and physiological processes, including synaptic transmissions (Yang, 2017). They are also are connected to inflamed tissue (Poet, 2001). Our work inhibiting FaNaCs further explores ligand-gated channels, as well as their involvement in tissue damage. We hope our research will prompt more experiments with DF2 in relation to the FaNaC channel. Future work could include testing DF2 in combination with additional activators to determine the response of multiple FMRFamides on synaptic transmission.

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REFERENCES

Badhwar, A., Weston, A. D., Murray, J. B., & Mercier, A. J. (2006). A role for cyclic nucleotide monophosphates in synaptic modulation by a crayfish neuropeptide. *Peptides, 27*(6), 1281-1290.

Brezden, B. L., Yeoman, M. S., Gardner, D. R., & Benjamin, P. R. (1999). FMRFamide-activated Ca2+ channels in lymnaea heart cells are modulated by "SEEPLY," a neuropeptide encoded on the same gene. *Journal of Neurophysiology, 81*(4), 1818-1826.

Cottrell, G. A. (2005). Domain near TM1 influences agonist and antagonist responses of peptide-gated na+ channels. *Pflügers Archiv - European Journal of Physiology*, *450*(3), 168-77.

Lingueglia, E., Deval, E., & Lazdunski, M. (2006). FMRFamide-gated sodium channel and ASIC channels: A new class of ionotropic receptors for FMRFamide and related peptides. *Peptides*, *27*(5), 1138-1152.

Mains, R. E., & Eipper, B. A. (2012). Peptides. In *Basic Neurochemistry* (8th ed.).

Mercier, A. J., Orchard, I., Tebrugge, V., & Skerrett, M. (1993). Isolation of two FMRFamide-related peptides from crayfish pericardial organs. *Peptides*, *14*(2).

Poët, M., Tauc, M., Lingueglia, E., Cance, P., Poujeol, P., Lazdunski, M., & Counillon, L. (2001). Exploration of the pore structure of a peptide-gated Na+ channel. *The EMBO journal*, *20*(20), 5595–5602.

Weiss, T., Kreissl, S., & Rathmayer, W. (2003). Localization of a FMRFamide-related peptide in efferent neurons and analysis of neuromuscular effects of DRNFLRFamide (DF2) in the crustacean Idotea emarginata. *European Journal of Neuroscience*, *17*(2), 239–248.

Yang, X., Niu, Y., Liu, Y., Yang, Y., Wang, J., Cheng, X., . . . Yu, Y. (2017). The nonproton ligand of acid-sensing ion channel 3 activates mollusk-specific FaNaC channels via a mechanism independent of the native FMRFamide peptide. *The Journal of Biological Chemistry*, *292*(52), 21662-21675.