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Temperature Dependence of DF_2's Effectiveness is Due to Temperature Sensitivity of Phosphatases

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ABSTRACT

The neuropeptide DRNFLRFamide (DF_2) increases synaptic transmission at the crayfish neuromuscular junction, but its function depends on temperature. The difference in DF_2's effectiveness in synaptic modulation may be due to different degree of dephosphorylation by protein phosphatases. Motivated by previous studies on temperature dependence of synaptic enhancement induced by DF_2, we hypothesized that (1) DF_2's function depends on phosphorylation and inhibition of phosphatases would increase DF_2's effectiveness. (2) At lower temperature phosphatases will be less active. Therefore, increase in DF_2's function at low temperatures is due to more phosphorylated proteins induced by inactiveness of phosphatases. We experimented with DF_2 and phosphatase inhibitors at both low and room temperatures. We determined the effectiveness of DF_2 by measuring Excitatory Postsynaptic Potential (EPSP) of extensor muscle cells in different solutions with five minutes of adjustment period using intracellular recording and repeated stimulation of every five seconds. Our data shows that DF_2's effectiveness increases at low temperature, and the effect of phosphatase inhibitors at low temperatures was not as prominent as that at room temperature. The results indicate that at low temperature, phosphatase has already been inhibited, and further inhibition with phosphatase inhibitors has no notable effect. This result supports our hypotheses, suggesting that the effectiveness of DF_2 increases in low temperature due to minimized function of phosphatase.

INTRODUCTION

DF_2 (DRNFLRFamide) is an FMRFamide-like peptide and a neuromodulator. DF_2 binds to a receptor and activates a G-protein cascade (guanine nucleotide-binding protein cascade). This cascade leads to the activation of protein kinases which phosphorylate their target proteins. Protein phosphorylation, in turn, potentiates the effect of DF_2 on synaptic transmission (Badhwar, Weston, Murray, & Mercier, 2006). As a result, DF_2 enhances Excitatory Postsynaptic Potential (EPSP) by increasing the amount of transmitter released from axon terminals (Badhwar, et al., 2006).

Dunn & Mercier (2003) demonstrated the close relationship between the efficiency of neuropeptides and temperature. DF_2 is more effective at 7-9 °C than at 15-17 °C (Friedrich, Quigley, Srivastava, Skerrett, & Mercier, 1994). However, little is known about the mechanism behind the temperature dependence of DF_2. Friedrich et. al (1994) suggested that DF_2's effects on synaptic transmission is enhanced because phosphatases are less functional at low temperatures.

Phosphorylation and dephosphorylation are processes that modulate the function of DF_2 (Abdul-Ghani, Kravitz, Meiri, & Rahamimoff, 1991). Phosphatases negatively modulate the degree of phosphorylated proteins in the cell. It has been shown to promote the degradation of organic phosphorous compounds (Pant, 2014) They reverse the function of the protein kinases, which add a phosphate group to their target proteins and lead to DF_2's long-lasting functions (Badhwar et al., 2006).

The effect of DF2 requires phosphorylation of the proteins, enhancing the amount of neurotransmitter released by changing the currents of potassium and calcium (Friedrich et al., 1998). However, phosphatases reverse the phosphorylated proteins to the initial state and thus, minimize the effect of DF_2.

According to Elias, Wieczorek, Rosenne, & Tawfik (2014), enzymes have universal enzymatic rate-temperature dependency. Every enzyme functions at its ideal temperature. Phosphatase, an enzyme, is thus affected by the temperature. At low temperatures, phosphatases' ability to remove the phosphate group from target proteins is inhibited. As a result, the abundance of phosphorylated proteins would further enhance DF_2's effect on synaptic transmission.

We hypothesized that phosphatases are less functional at low temperatures, and it potentiates the effect of DF_2 on synaptic enhancement by slowing down the decay of phosphorylated target proteins. We measured DF_2's effectiveness by observing the change of EPSP amplitude. DF_2 increased the EPSP amplitude at both room and low temperature. To confirm that phosphatases are less functional at low temperatures, we compared the EPSP amplitudes with and without phosphatase inhibitors at low temperatures. Our data suggested that at low

temperature, phosphatases are less functional, and further inhibition of phosphatases does not affect the activity of DF_2. Although our data is not statistically significant, it tentatively supports our hypothesis. This research will provide a better understanding of the role of phosphatases in synaptic enhancement and the mechanism behind the increase in DF_2's effectiveness at low temperatures.

MATERIALS AND METHODS

Preparation

Crayfish were maintained and provided by Grinnell College. The crayfish were first anesthetized by being placed in an ice bath for 15 minutes. We experimented on extensor muscle cells and the nerves in the dorsal part of the crayfish tail. To isolate the muscles, we separated the tail from the crayfish by cutting the crayfish between the tail and thorax using scissors. We removed the ventral surface of the tail. To make the extensor muscles and the nerves more visible, we scooped out excess muscles and gut. We pinned the dorsal surface of the tail facing down in the dish. We submerged the tail with normal crayfish Ringers' solution. The crayfish tail always rested in the normal crayfish Ringers' solution unless we changed to different solutions.

Solutions

We used normal crayfish Ringers' solution (5.4mM KCl, 196mM NaCl, 2.6mM $MgCl_2$-$6H_2O$, 10mM HEPES, 13.5 mM $CaCl_2$-$2H_2O$, pH 7.4) to bath the muscle cells and maintain their normal function. The preparation was bathed in normal crayfish Ringers' unless switched to a different solution. We applied 50nM DF_2 in Ringers' solution as suggested by Weiss, Kreissl, & Rathmayer (2003) and 100µL of phosphatase inhibitors cocktail solution (2.5µM Bromotetramisde, 0.5µM Cantharidin, 1µM Calyculin A) at room temperature (RT) (19-20°C) and at low temperature (LT) (7-10°C). We adjusted the temperature by putting the solution in the freezer 15 minutes prior to the experiment. We put cubes of ice-Ringers' inside the solutions to maintain the temperature and measured the temperature near the muscle cell with a digital thermometer.

Intracellular EPSP Recording

We made suction electrodes and recording electrodes using glass capillary tubes pulled with PUL-1 World Precision Instruments puller. We filled the recording electrode with 3M KCl. The acceptable resistance for the recording electrodes was between 4 and 25 MΩ. For the suction electrode, we sanded the tip of microelectrodes. After sucking up the nerve, we stimulated the nerve with a suprathreshold stimulus using a Grass stimulator (f=0.2Hz). We measured the membrane potential using an amplifier for 10 minutes and calculated the EPSP by subtracting the minimum from the maximum value following the stimulus artifact. The data was collected with Powerlab and the lab chart software with mains filter on.

Data Analysis and Statistical Test

We calculated percentage change for each EPSP amplitude using the formula:

$$\% \text{ Change} = \frac{(EPSP\ treatment)}{EPSP_{control}} \times 100\%$$

RESULTS

We studied the effect of DF_2 at different temperatures with phosphatase inhibitors. For each chemical, we recorded EPSPs using intracellular recording for 10 minutes after adding the chemical for 5 minutes and calculated the percentage change using the formula. (See *Methods*)

To confirm that DF_2 increases synaptic efficacy (Skerrett, Peaire, Quigley, & Mercier, 1995), we compared EPSP percentage change in control experiments (n=6) to those treated with DF_2 (n=5) at room temperature.

For our control experiment, we measured EPSP amplitude in normal crayfish Ringers' solution. We calculated our baseline data to compare with other sets of experiments. We used the baseline data of control experiment as the benchmark for all graphs we show.

On the same crayfish, we changed the solution to that with addition of DF_2. We let the solution infiltrate the cells for five minutes. On average, EPSP amplitude increased by 141% under the presence of DF_2 at room temperatures. The EPSP percentage with DF_2 at room temperatures were also higher than that of control until 535 seconds (Fig.1). The result reconfirmed the potentiating role of DF_2 in synaptic transmission.

To examine DF_2's temperature sensitivity, we compared percentage of EPSP change induced by DF_2 at room temperature (n=5) and low temperature (n=2) over time.

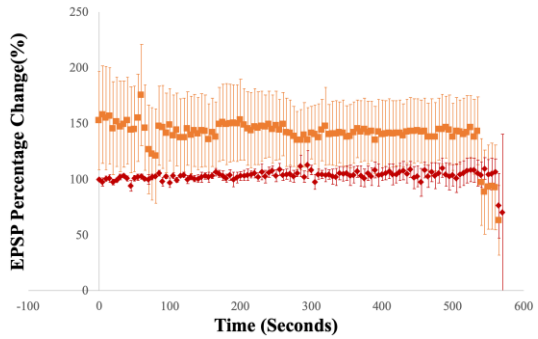

Fig.1*:* The orange squares represent percentage change of EPSP amplitude in DF_2 solution at room temperatures over time (n=5), and the red diamonds represent percentage of EPSP change for control experiment (n=6). Each marker represents mean of two to five data. Error bars are the standard errors of the mean.

We used the data from the previous experiment on EPSP amplitude induced by DF_2 at room temperature. On the same crayfish, we switched the DF_2 solution into that of low temperature (with the average of 10.2°C for first trial and 11.2°C for second trial). We modulated the temperature using Ringers' ice cubes for the first trial and we put the DF_2 solution beaker in the freezer for the second trial. We maintained the temperature for both trials by using Ringers' ice cubes and measured the temperature near the muscle cell throughout the trials. The results suggest that compared to baseline data, the effect of DF_2 at low temperature is not as prominent as that of room temperature. While at room temperature, the mean value of percentage change was 141% (Fig.1), that of low temperature was 120% (Fig.2). However, if we consider the initial drop of EPSP at low temperature by 88% (Friedrich et al., 1994), through calculation, DF_2's effectiveness actually increases by 450% at low temperature. Therefore, although our data seems to contradict Friedrich et al.'s (1994) finding that the effect of DF_2 is enhanced at low temperature, our results still support the same finding. (See *Discussion*)

To determine the role of phosphatases in the synaptic enhancement induced by DF_2, we compared percentage change of EPSP amplitude induced by phosphatase inhibitors and DF_2 (n=2) to that of DF_2 only (n=5) both at room temperature.

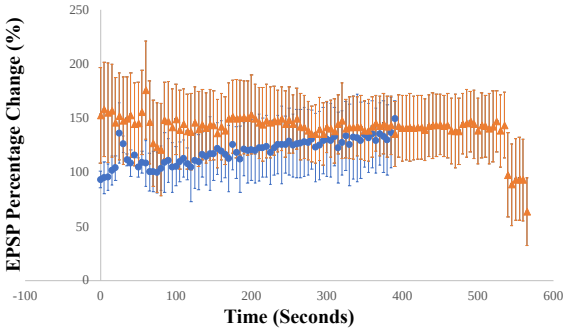

Fig.2: The orange triangles represent the percent change of EPSP amplitude under the condition of DF_2 at room temperature over time (n=5), and the blue circles represent the percent change of EPSP amplitude induced by DF_2 at low temperature over time (n=2). Each marker represents mean of each data set. Error bars are the standard errors of the mean.

We used data from the experiment on EPSP amplitude induced by DF_2 at room temperature. We used the same crayfish and added phosphatase inhibitors cocktail using a micropipette into the DF_2 solution. We had two trials with different methods of mixing chemical for this experiment. For the first trial of the experiment, we injected the phosphatase inhibitors cocktail with a micropipette and dispersed the cocktail by pumping repeatedly. For the second trial of the experiment, we followed the same procedure to add phosphatase inhibitors but mixed the solution by stirring with forceps. For this trial, we let the solution infiltrate the muscle cells for five minutes. Under the treatment with the combination of phosphatase inhibitors and DF_2, the EPSP amplitude increased by 260%. This was a greater change than when treated with only DF_2 up to 170 seconds (Fig.3). After 170 seconds, the percentage increase of EPSP amplitude dropped abruptly to average of 84% (Fig.3).

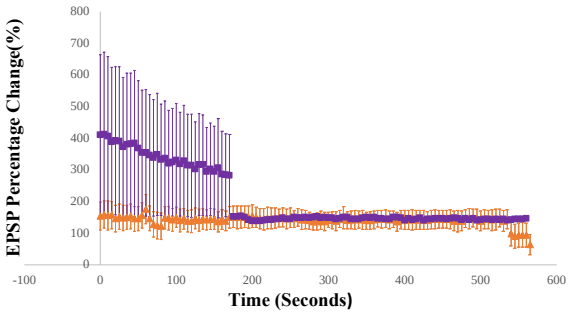

Fig.3*:* The purple squares refer to the percentage change of EPSP amplitude at room temperature induced by phosphatase inhibitors cocktail and DF2 solution over time (n=2). The orange triangles represent the percentage change of the EPSP amplitude in the solution treated with DF_2 at room temperature over time (n=5). Each marker represents mean of two to five data. Error bars are the standard errors of the mean.

To verify the change in effectiveness of phosphatases at low temperature, we compared the percentage change of EPSPs in DF_2 with (n=1) and without (n=2) phosphatase inhibitors at low temperature.

We used the same data on DF_2 low temperature in Fig.2 to compare. After measuring the EPSP at low temperature, we used a micropipette to pump the phosphatase inhibitors into the solution. The solution's temperature at the time of injection was 9.7°C and it was maintained throughout the trial with cubes of iced-Ringers'. We let the solution sit and kept stimulating to make sure we did not miss out any possible change in EPSP. The data shows that the sudden jump in EPSP amplitude is relatively big, up to 153% but it can only maintain for 50 seconds before dropping back to normal. Afterwards, the EPSP amplitude constantly increased until the end of the trial, averaging 132% (Fig.4)

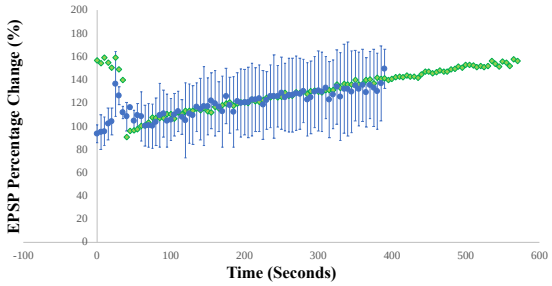

Fig.4*:* The green diamonds represent change of EPSP amplitude in DF_2 and phosphatase inhibitor cocktail solution at low temperature (n=1), and the blue circles represent change of EPSP amplitude in DF_2 solution at low temperatures (n=2). Each marker represents mean of two to five data. Error bars are the standard errors of the mean.

Compared to the control data, all of the experiments show an increase in EPSP amplitude (Fig.5). Since the extreme change by phosphatase inhibitors lasted a short amount of time, we separated data into two groups: change before the sudden drop and total change over time. The data of cocktail inhibitor and DF_2 before dropping had the highest average EPSP percentage change (260%), and the second highest group was the data of cocktail inhibitor and DF_2 induced group before drop at low temperature (153%). This data indicates that at experimental temperatures, the effect of phosphatase inhibitors is prominent.

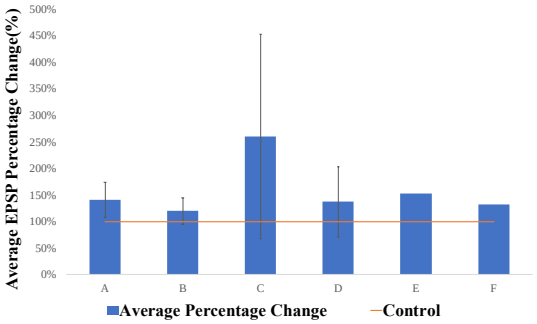

Fig5*:* The bar graph illustrates average change of EPSP amplitude of all experiments we measured. (A) DF_2 induced EPSP at room temperature (n=5); (B) DF_2 induced EPSP at low temperature (10-12°C) (n=2); (C) DF2 induced EPSP with phosphatase inhibitors at room temperature before the drop (n=2); (D) DF2 induced EPSP with phosphatase inhibitors overall (n=2); (E) DF2 induced EPSP with phosphatase inhibitors at low temperature before the drop (9.7°C) (n=1); (F) DF_2 induced EPSP with phosphatase inhibitors at low temperature overall (n=1). The orange line at 100% is the control data baseline (n=6).

DISCUSSION

Our data tentatively support our hypothesis that DF_2's activity is more profound at low temperature because phosphatases are less active. We confirm that (1) DF_2 increases synaptic activity at room temperature and low temperature. (2) EPSP amplitude induced by DF_2 at low temperature is enhanced by roughly 450% compared to that of room temperature. (3) At low temperature, phosphatases are less effective in negatively modulating synaptic transmission induced by DF_2. Therefore, our result explains the conclusion on the study of temperature-dependence of synaptic modulation by DF_2 on crayfish (Friedrich et al., 1994).

Our results reaffirm the potentiating effects of DF_2 in both room temperature and low temperature. However, for the sets of experiments to compare DF_2's effectiveness at low temperature and room temperature, our data contradicts Friedrich et al. (1994). We expected that at low temperature, EPSP amplitude would be higher than that of room temperature, but our results show that the average EPSP amplitude is decreased by 19%. However, at low temperature, EPSP amplitude is decreased by 88% (Friedrich et al., 1994) and DF_2 helps compensate for the decreased synaptic efficacy. Our calculation confirms that although the initial results might seem counterintuitive, the effect of DF_2 on synaptic transmission is 450% higher at low temperature than that at room temperature.

We also determined the modulating role of phosphatase and its temperature dependency by comparing the EPSP amplitude after adding the phosphatase inhibitors cocktail at room temperature and at low temperature. While for room temperature, the effects on EPSP amplitude induced by DF_2 is prominent as the percentage change in EPSP amplitude increases by

260% and is maintained by around three minutes. At low temperature, the effect of the inhibiting cocktail is reduced, as EPSP amplitude only increases by 153% for 30 seconds before dropping back. The results indicate that before adding the cocktail, phosphatases have already been inhibited by the drop in temperature.

Another interpretation of this data is that the abrupt changes in EPSP with phosphatase inhibitors were due to the stimulation becoming subthreshold for one or more axons in the nerves. This could reflect the unbeknownst effect of phosphatase inhibitors on the threshold in the nerve. Further research can look into other possible effects of the phosphatase on synaptic efficacy other than inhibiting phosphatase.

However, a variety of limitations affect the reliability and validity of this study. Firstly, our lab data is inconsistent because of the lack of lab sessions and hours and as a result, our result is not statistically significant. Secondly, temperature is a hard variable to maintain, so the margin of error for low temperature in our experiment is sometimes out of the pre-determined parameter. Since phosphatase enzyme is very sensitive to change in temperature, the inconsistency in temperature between experiments might hinder our final results. Thirdly, we were unable to gather enough data points for some experiment sets. For example, we only have one suitable data points for the effecs of DF_2 after adding the cocktail at low temperature. Future research can conduct more experiments to provide more reliability to the final data. Finally, because we use normal crayfish Ringers' with Ca^{2+}, the muscle usually twitched a lot and electrodes were frequently broken or slipped out of the targeted cell. Future research can consider using lower concentration of Ca^{2+} crayfish Ringers' to prevent or reduce muscle twitching and therefore, maintain a consistent result throughout the experiment.

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