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FRMFamide Combined with IBMX Decreases EPSP Amplitude.

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ABSTRACT

The aim of this experiment was to explore the effects of the neuropeptide FMRFamide (FMRFa) on the crayfish neuromuscular junction (CNJ). In previous experiments, DF_2, a FMRFa-like peptide, has been shown to increase neurotransmitter (NT) release at the CNJ. We hypothesized that, like DF_2, FMRFa would also potentiate the release of NT and thereby increase excitatory postsynaptic potential (EPSP) amplitude. We also hypothesized that IBMX, a phosphodiesterase inhibitor, would be needed to potentiate the excitatory effects of FMRFa, which it has been shown to do with DF_2. To test these hypotheses, we exposed the crayfish to both a high and low Ca^{2+} Ringer's solution and to various treated solutions. The first treated solution contained just FMRFa, the second IBMX, and the third both FMRFa and IBMX. We then measured NT release by EPSP amplitudes, recorded from postsynaptic muscle cells via intracellular recording. Our data showed a trend that in both low and high Ca^{2+} Ringer's solution, FMRFa did increase neurotransmitter release, supporting our first hypothesis. Our second hypothesis was rejected, for our data showed that in a FMRFa and IBMX treated solution average EPSP amplitude decreased.

INTRODUCTION

Phe-Met-Arg-Phe-NH_2, or FMRFa, is a neuropeptide from a family of FMRFa-related peptides called FaRPs. DF_2, belonging to this family of FMRFa-like peptides, has been shown to increase neurotransmitter (NT) release at the crayfish neuromuscular junctions (CNJ) (Skerret *et al* 2005). Concerning the excitatory effects of DF_2, Friedrich *et al* (1998) suggest that protein kinase C is involved, while Badhwar et al (2006) posit involvement of protein kinase A and G. Additionally, Noronha and Mercier (1995) claim that Ca^{2+}-calmodulin dependant protein kinases are necessary for this excitatory effect of DF_2. It has also been suggested that nitric oxide (NO), a retrograde messenger, is involved in FMRFa's ability to increase NT release. Rõszer *et al* (2006) suggest that FMRFa is a substrate source of NOS synthase in the gastropod nervous system, because it enhances NO liberation. Our experiment explored the relationship between FMRFa and DF_2, a relationship that has not fully been addressed. Our experiment specifically tested to see if FMRFa would increase NT release at the CNJ, as DF_2 has previously been shown to do.

We hypothesized that FMRFa would increases NT release at the *Procambarus clarkii* neuromuscular junction, increasing EPSP amplitude. We also hypothesized that IBMX, a phosphodiesterase inhibitor that slows the break down of cAMP and cGMP, would be needed to potentiate the excitatory effects of FMRFa (Badhwar *et al* 2006). To test our hypotheses we exposed the superficial dorsal extensor muscles in the tail of the crayfish to various solutions and measured their EPSPs. The first solution contained a low Ca^{2+} Ringer's solution, while the test solution was treated with FMRFa, then IBMX, followed by a combination of both. We repeated this process in a high Ca^{2+} solution to see if calcium was needed to potentiate the effects of FMRFa.

While our data was statistically insignificant, our results did indicate a trend towards slightly increased EPSP amplitude in low Ca^{2+} Ringer's solution with FMRFa present. Our data was, however, statistically significant in the high Ca^{2+}, showing an increase in EPSP amplitude. Overall, our data supported our first hypothesis. Our second hypothesis that IBMX was needed to potentiate the effects of FMRFa was rejected. Our data showed that IBMX decreased the effects of FMRFa, suggesting that the excitatory effects of IBMX on DF_2 are not analogous to those on FMRFa.

MATERIALS AND METHODS

Saline Solutions

The low Ca^{2+} Ringer's saline solution was comprised of KCl 5.4 mM, NaCl 196 mM, $MgCl_2.6H_2O$ 7.1 mM, Na Hepes Buffer 10 mM, and $CaCl_2.2H_2O$ 6.0 mM, adjusted to a pH of 7.4. This solution was changed every 15 minutes. We then diluted the same solution with a 100 mM stock solution of FMRFa, to a concentration of 100 μM. To make our second test solution, we diluted the original Ringer's solution with a 100 mM stock solution of IBMX to a concentration of 100μM. This was done

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with a micropipettor. The third test solution was the Ringer's solution diluted down to a 100µM concentration of FMRFa stock solution and a 100µM concentration of IBMX stock solution. We then prepared the first and third test solution with a high Ca^{2+} Ringer's solution comprised of KCl 5.4 mM, NaCl 196 mM, $MgCl_26H_2O$ 2.6 mM, Sodium Hepes Buffer 10 mM, and $CaCl_22H_2O$ 13.5 mM with pH of 7.4.

Experimental Proceedings
The preparations were changed daily, and exposed to the solution for at least ten minutes before recording. We exposed our preparation to the FMRFa solution and IBMX solution on different days. Additionally, the preparation was always exposed to the FMRFa + IBMX soltuion directly after it was exposed to either the FMRFa or IBMX solution.

Crayfish Specimen
The crayfish were purchased from Carolina Biological Supply Company (North Carolina, U.S.A.). The crayfish were kept in an ice bath before dissection. We then cut alongside the tail's ventral surface and removed the swimmeretes, intestines, and abdominal muscles, leaving behind the dorsal superficial extensor muscle. We pinned the crayfish by two pins in a 200ml dissection dish, with silicone elastomer. We then poured the various saline solutions over the crayfish into the dish.

Electrophysiology
We used two electrodes, a recording microelectrode and nerve-stimulating suction electrode. We pulled apart the 1.2mm microelectrode with a PUL-1 micropippette puller. We then filled it with 3 M KCl, rinsed it in a saline solution, and placed it on the micromanipulator. The resistance of the microelectrodes were consistently measured to be >20 MΩ.

Nerve Stimulation and Data Recording
The suction electrode was connected to a stimulator, which stimulated the captured pre-synaptic nerve. It was stimulated with single pulses at a frequency of 0.5 Hz and at the lowest voltage possible to measure an EPSP. Using the micromanipulator we then placed the electrode in the muscle most lateral to the captured nerve to record the signals in the post-synaptic muscle cell. These signals were passed through an amplifier and recorded with the computing program Scope.

RESULTS

To see if FMRFa increased NT release we compared the EPSP amplitudes recorded from the crayfish tail while in a high Ca^{2+} Ringer's solution and while in a solution diluted with FMRFa. The differences in the mean EPSP amplitudes were statistically significant (p =0.003), showing us that FMRFa increased the EPSP amplitude. FMRFa increased the EPSP amplitude on average by 6.6 mV, which supports our hypothesis regarding the excitatory effects of FMRFa (Figure 1).

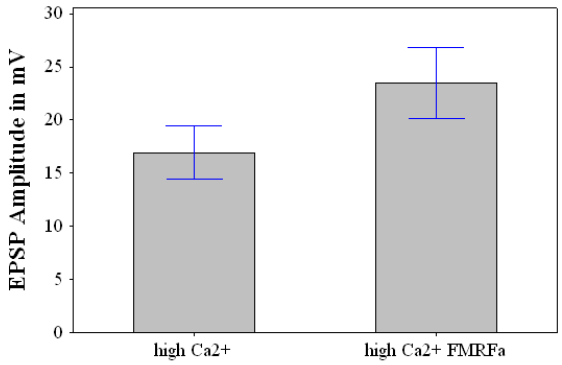

Figure 1. Average EPSP amplitude before and after exposure to FMRFa. High Ca^{2+}: n=5, error bar indicates standard error of 2.5. High Ca^{2+} + FMRFa: n=4, error bar indicates standard error of 3.3. The p value is 0.003.

We then tested the effects of FMRFa in a low Ca^{2+} solution. The average EPSP amplitude recorded in the control solution was 12.6 mV, while it increased to an average of 13.5 mV when FMRFa was added. These results, while showing a trend that FMRFa increases NT release, were statistically insignificant (p=0.052). To test the hypothesis that IBMX would potentiate the effects of FMRF, we added both IBMX and FMRFa to the control solution. Our results show that IBMX decreased the effects of FMRFa on average by 3.8 mV, causing us to reject our hypothesis (Figure 2). This result was statistically significant (p<0.001), suggesting that IBMX decreases the EPSP amplitude when added with FMRF in a low Ca^{2+} solution.

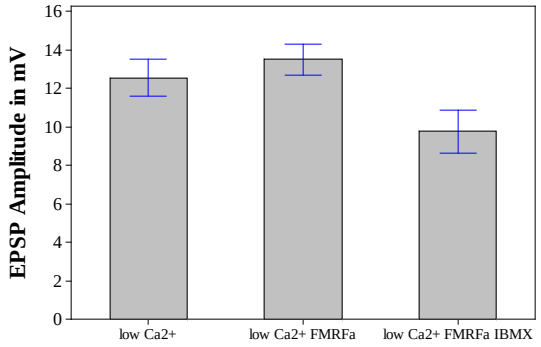

Figure 2. Average EPSP amplitude before and after exposure to FMRF and IBMX. Low Ca^{2+}: n=18, error bar indicates standard error of 1. Low Ca^{2+}+ FMRFa: n=13, error bar indicates standard error of 0.8. Low Ca^{2+}+FMRFa+IBMX: n=14, error bar indicates standard error of 1.1. $p<0.001$ when comparing low Ca^{2+} to low Ca^{2+}+FMRFa+IBMX, and when comparing low Ca^{2+}+FMRFa to low Ca^{2+}+FMRFa+IBMX. $p=0.052$ when comparing low Ca^{2+} to low Ca^{2+}+FMRFa.

After our second hypothesis was rejected, we tested to see if IBMX would have the same negative effects if applied alone without FMRFa. To test this we compared the EPSP amplitude in a low Ca^{2+} solution to the EPSP amplitude in a low Ca^{2+}+IBMX solution. While the p value was 0.552, we saw that IBMX increased the EPSP amplitude on average by 2mV, n=4.

DISCUSSION

Our first hypothesis was that FMRFa would increase EPSP amplitude. Overall, our data supported a trend that FMRFa increases NT release (Figure 2). While we did see this trend, our data revealed unclear results. For example, in low Ca^{2+} our data was statistically insignificant, while it was significant in high Ca^{2+}. In addition to this, our results varied from day to day, showing how biological variability affected our experiment. Our second hypothesis was that IBMX would potentiate the effects of FMRFa. Our data rejected this hypothesis, suggesting that IBMX in combination with FMRFa does not enhance FMRFa's effects, but rather reverses them (Figure 2).

Our experiment raises questions of the role of IBMX on NT release. Previous studies suggested that IBMX enhanced the effects of DF_2 (Badhwar *et al.* 2006; Morley *et al.* 2009). Despite the fact that DF_2 is a member of the FMRFa family, our data suggests that IBMX does not have the same effects on FMRFa that it does on DF_2. This observation is important, for the target receptor of FMRFa is still unclear. Therefore, there is room for further study as to whether or not FMRFa truly increases NT release, and if so by what mechanism it achieves this. Rőszer *et al* (2006) suggest that FMRFa is a substrate source of nitric oxide (NO), which could suggest that NO acts as a signaling molecule, increasing NT release. To test this question, L-NAME, an inhibitor of NO synthase, could be used to see whether the effects of FMRFa could be halted or reversed, suggesting that NO is involved in its excitatory effects.

Our data was the most unreliable in the high Ca^{2+} because the high concentration triggered action potentials that jeopardized our data collection. Our data, however, was statistically significant in high Ca^{2+}, showing the largest difference between the EPSP amplitude of the control and that of the FMRFa solution (Fig. 1). Further testing is required to see how calcium affects the possible excitatory effects of FMRFa. Further tests could include using a calcium chelator, such as EGTA, to see if the observed excitatory response of FMRFa would be reversed by lower concentrations of Ca^{2+}. This could suggest a possible target receptor for FMRFa.

In testing for the direct relationship between the EPSP amplitude of low Ca^{2+} and low Ca^{2+}+ IBMX, our data, although statistically insignificant (p=.552), showed that IBMX alone increased the EPSP amplitude by 2mV. This data was not as informative as our other data, because there were only four recordings taken to test the effect of IBMX. If these results were supported by future tests, they would suggest that FMRFa reverses the excitatory effects of IBMX. This would give us valuable information regarding the mechanism by which FMRFa increases NT release.

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