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Protein Kinase C is Partially responsible for the Effects of NF_1 in Sustaining EPSP Amplitude Increase

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ABSTRACT

We used crayfish neuromuscular preps to examine the role of protein kinases in general and Protein Kinase C (PKC) in particular in the effects of sustaining EPSP amplitude increases induced by NF_1. We used intracellular recordings to measure changes in EPSP amplitudes over time under the effects of different protein kinase inhibitors and NF_1 as compared to baseline measurements taken before the exposure to drugs/NF_1. We found that preps exposed to NF_1 experienced a sustained increase in EPSP amplitude relative to their baseline measurements. We also found that while preps exposed to NF_1 with a PKC inhibitor experienced an initial increase in EPSP amplitude similar to that of preps exposed to NF_1 alone, they experienced a subsequent drop in EPSP amplitude relative to baseline levels, indicating that PKC is involved in the effects of NF_1 in that it plays a role in sustaining EPSP amplitude. Preps exposed to NF_1 and a general protein kinase inhibitor experienced a greater drop in EPSP amplitude than those exposed to NF_1 and a PKC inhibitor, indicating that PKC is not the only protein kinase involved in the effects of NF_1, inviting further research into the roles of other protein kinases in NF_1's effects. Our research fills a gap in the current literature, as although previous studies indicated the role of PKC in the effects of DF_2, a similar FMRFamide, we did not find any existing literature on the role of PKC in the effects of NF_1.

INTRODUCTION

Neuromodulators are substances which affect communication at the synapse (Nadim & Butcher, 2014). We know from previous studies that two neuromodulators, FMRF-amide-like peptides NF_1 and DF_2 have both been isolated in crayfish (Mercier et. al, 1993). Skerrett et al (1995) found that both DF_2 and NF_1 cause synaptic enhancement and EPSP amplitude increase in crayfish deep extensor muscles.

Protein Kinase C is an enzyme family. They respond to DAG (diacylglycerol), and Ca^{2+} (Huang,1989). Protein Kinase C (aka PKC) mediates the phosphorylation of certain cellular proteins. It plays a role in many signal transduction events in cells and is a member of the serine threonine kinase family (Mochly-Rosen & Kauvar, 1998). In the nervous system, the activation of Protein Kinase C relates to the regulation of neurotransmitter release, ion channels, and neuroplasticity (Huang, 1989). The binding of DF_2 to a GPCR (G-coupled protein receptor) leads to a signal cascade which activates PKC (Newton & Gold, 2008).

Research has demonstrated that PKC plays a role in synaptic enhancement by DF_2 (Freidrich et al, 1998). Researchers studying the role of PKC in synaptic enhancement by DF_2 previously concluded that PKC is responsible for the effects of DF_2 in that PKC limits the initial amplitude of EPSPs, but allows EPSPs to last longer (Freidrich et al, 1998). However, there appears to be a gap in the literature regarding the role of protein kinases and protein kinase C in the effects of NF_1. Thus, we intend to provide insight into the role protein kinases, especially protein kinase C in particular, play in causing the effects of the FMRF-amide like neuromodulator NF_1.

As NF1 and DF2 are members of the same family of peptides, and research has shown them to have similar effects on EPSPs in crayfish (Skerrett, et al. 1995), we predict that PKC plays a similar role in NF1 as it does in DF2. Therefore, we predicted that PKC will also sustain the EPSP amplitude enhancement by NF1 in the crayfish deep extensor muscles.

Our results supported our hypothesis that PKC does play a role in sustaining the prolonged increasing effect of NF_1 on EPSP amplitude$_1$. However, PKC is not the only protein kinase that helps sustaining the increasing effect of NF_1 on EPSP amplitude.

MATERIALS AND METHODS

Organisms and Preparation

We performed experiments on nerve muscle preps taken from adult red crayfish (*Procambarus clarkii*) obtained from Carolina Labs. Prior to dissection, crayfish were kept in an ice bath. To expose the extensor muscles, we made a diagonal cut through the connection between the crayfish's thorax and its tail, separating the thorax and

the tail. We then turned the crayfish abdomen dorsal side up and separated the shells of the ventral abdomen from the dorsal shells by using a pair of scissors to cut along the side of its abdomen. After lifting up the abdominal shell, which was still connected to the tail, we cut it off the tail completely, exposing the muscles underneath. We used a thumb to scrape off the muscle mass and expose the extensor muscles we were testing.

After dissecting the crayfish, we pinned the prep ventral side up in a glass dissection dish; one needle on the posterior side of the tail and the other two on two separate sides of the anterior part of the tail.

Equipment and Setup

To record EPSPs, our team used borosilicate glass recording microelectrodes with an outside diameter of 1.2 mm and a tip diameter of <1μm filled with 3mol 1^-1 potassium chloride with a resistance of 5~20 MΩ. They were connected to a microelectrode holder attached to a microelectrode manipulator. We used a 1600 A-M Systems DC amplifier, a Power Lab, and a computer running Lab Chart by AD Instruments to record the membrane potentials. We used a suction microelectrode made from borosilicate glass with an outside diameter of 1.2 mm connected to syringe to apply suction to isolate and stimulate crayfish nerves. We used the DS9 Simulator by Astro-Med Inc to stimulate the axon sucked in by the suction electrode.

Intracellular Recording and Data Collection

To take baseline measurement, we placed each prep in normal crayfish physiological saline (5.4μM KCl,196 μM NaCl, 26μM $MgCl_2 \bullet 6H_2O$, 10 μM HEPES, 13.5 μM $CaCl_2$,pH=7.4) prepared by our lab. After isolating a nerve using a suction microelectrode, and inserting the recording microelectrode into the appropriate muscle, we stimulated the nerve, and recorded baseline EPSPs.

After recording baseline EPSPs for each crayfish, we used a solution switching device to replace the normal crayfish physiological saline for crayfish physiological saline containing NF1, a LY 333 531, or staurosporine or NF1 with staurosporine or LY 333 531(which were prepared by our lab with the concentrations noted above). EPSPs we recorded using the same procedures used when taking baseline EPSPs. We began recording 1-5 minutes after switching solutions.

We used the data we collected of EPSP measured in crayfish physiological saline with no additional chemicals as our control group and everything else as the comparison group. For the data we collected, we compared EPSP measured in crayfish physiological saline with NF1 alone, staurosporine alone, LY 333 531 alone, NF1 with staurosporine, and NF1 with LY 333 531 with EPSP measured in crayfish physiological saline with no additional chemicals. We presented the comparison groups data as percentage in respect to the control group data.

We measured the EPSPs for 10-20 minutes with 5 seconds interval for each control group for each crayfish. For the comparison groups, we measured the EPSP with 5 seconds intervals for 20 minutes of NF1 alone, Staurosporine alone, LY 333 531 alone, NF1 combined with Staurosporine, NF1 combined with LY 333 531. The concentration for NF1 was 50 nM, for Staurosporine was 2.15 μM, and for the PKC inhibitor was 10 μM.

For the raw data we collected, we took only the difference between the maximum and minimum membrane potential without the artifact using Microsoft Excel to get the EPSP amplitude. Then we treated the control group data as 100%, or 1. After that, we compared the comparison group data to the control groups data and present as the percentage in respect to the control group data. We graphed the data using the percentage as the vertical axis and time as the horizontal axis.

RESULTS

To examine the role of PKC on the effects of NF_1, we exposed crayfish neuromuscular preps to crayfish physiological saline containing NF_1 alone, NF_1 and staurosporine together, staurosporine alone, LY 333 531 alone, and NF_1 and LY 333 531 together. We used intracellular recording to record EPSPs. For each prep, we recorded EPSPs stimulating every 5 seconds for 10-20 minutes prior to exposure to Staurosporine, LY 333 531, and NF1 as the baseline, and for an additional 10-20 min stimulating every 5 seconds following the exposure to the three substances mentioned to observe their effects on EPSPs over time. We calculated the mean value of our baseline EPSP since it was mostly the same number and the mean value eliminated variability in the data collecting process. We compared the EPSPs with the influence of substances to the mean value of the baseline in percentage. Then we computed and compared these data with respect to time and came up with the graphes being shown below.

The Effects of Staurosporine on EPSPs

We found that when we added Staurosporine, a protein kinase inhibitor, into the crayfish, the EPSPs immediately started to drop (figure 1). The EPSPs dropped at a relatively rapid rate in the first ten minutes when staurosporine was added, then the EPSPs stayed at a relatively steady level and dropped at a much slower rate at the second ten minutes. As shown in Fig. 1, in the first ten minute period, the amplitude of the EPSPs affected by

Staurosporine compared to the baseline EPSP dropped from about 75% to 15%. In the second ten minutes period, the EPSP affected by Staurosporine compared to the baseline EPSP dropped from 15% to 10%.

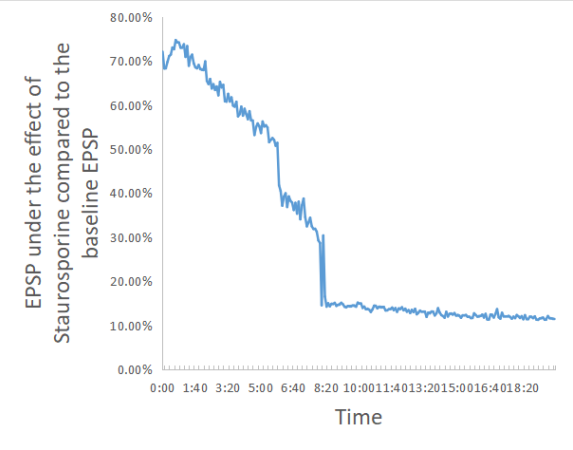

Fig. 1. EPSP under the effect of Staurosporine compared to the baseline EPSP in percentage with respect to time.

The Effects of LY 333 531 on EPSP

We found that when we added LY 333 531, the protein kinases C inhibitor, into the crayfish, the EPSP immediately experienced a slight drop. As shown in Fig. 2, the EPSP dropped to around 80% compared to the baseline EPSP and stayed around this value for the ten minutes period. The EPSP stayed steady after the initial drop.

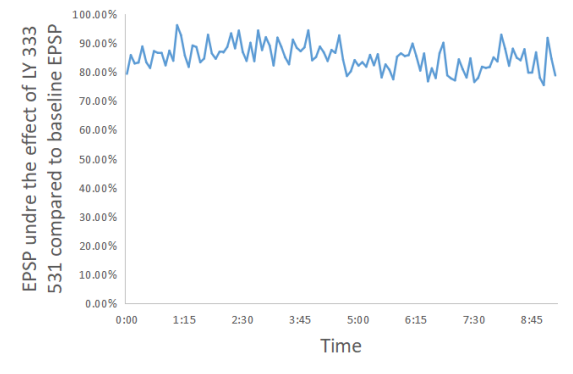

Fig. 2. EPSP under the effect of LY 333 531 compared to baseline EPSP in percentage with respect to time.

The Effects of NF1 on EPSP

We found that when we added NF1, a neuromodulator from the FMRFamide family, the EPSP did not immediately started to raise. As shown in Fig. 3, in the first five minutes, the EPSP stayed fairly steady at around 120 to 140%. However, after the first five minutes, the EPSP started increasing very fast. It went from 140% to almost 200%.

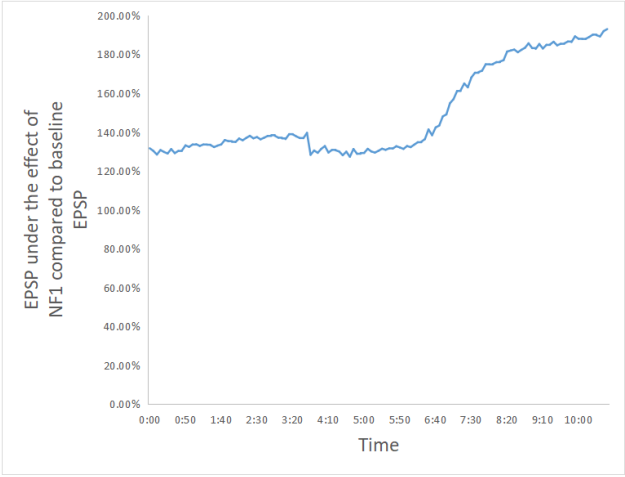

Fig. 3. EPSP under the effect of NF1 compared to baseline EPSP in percentage with respect to time.

The Effects of Staurosporine Combined with NF1

We found that when we added Staurosporine combined with NF1, the EPSP experienced an increase from 100% to 140% in the first five minutes (Fig. 4.). Then it started dropping from 140% to 20%. We believed that the abrupt rise and drop of EPSP at around 1:40, 4:10, and 8:20 was due to the fact that we sucked in two axons in the suction electrode at the same time. The suction electrode stimulated one axon before 1:40 but started stimulating two axons at the same time from 1:40 to 4:10, then the second axon dropped out after 4:10, and got sucked in again at 8:20. These stimulation and dropping out of the second axon caused the rise and drop of the EPSP. However, if we removed these distractions, we could still see a gradual drop of EPSP. Therefore, we believed that the effects of Staurosporine combined with NF1was causing the EPSP to drop.

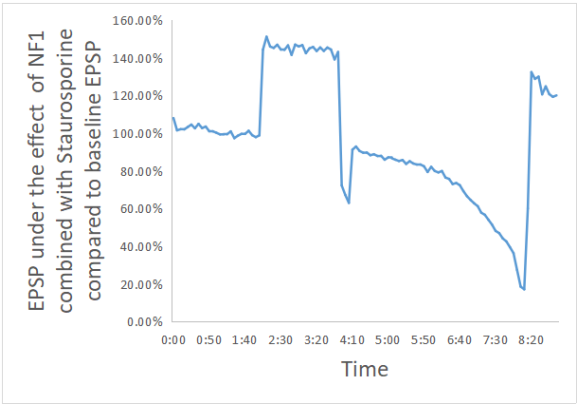

Fig. 4. EPSP under the effect of NF1 combined with Staurosporine compared to baseline EPSP in percentage with respect to time.

The Effects of LY 333 531 Combined with NF1

We found that when we added LY 333 531 combined with NF1, the EPSP immediately went to 200% (Fig. 5). As soon as it reached 200%, it started dropping to baseline level in ten minutes.

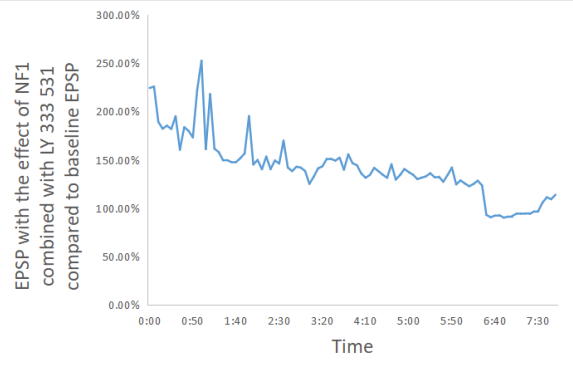

Fig. 5. EPSP under the effect of NF1 combined with LY 333 531 compared to baseline EPSP in percentage with respect to time.

Our data supported our hypothesis that protein kinase C does play a role in sustaining NF1's increasing effect on EPSP amplitude. When protein kinase C is inhibited, although EPSP amplitude did experience an initial increase like it did with solely NF1, this increasing effect did not sustain over time and the EPSP amplitude dropped back to baseline level instead of staying at a higher level. However, we found that compared to inhibiting protein kinase C alone, inhibiting all protein kinases made the EPSP amplitude drop below the baseline a lot when NF1 was induced.protein kinase C. Therefore, protein kinase C is not the only protein kinase that help sustain the increased EPSP amplitude when NF1 was induced. Other protein kinases also play a role in this sustaining effect.

DISCUSSION

The results of our study supported our hypothesis that PKC would play a similar role in the effects of NF_1 as it does in the effects of DF_2 in that it sustains the increasing effect of NF1 on EPSP amplitude over time. Our data revealed that preps exposed to NF_1 experienced about a 200% increase in EPSP amplitude. However, the preps exposed to NF_1 & LY 333 531 experienced a subsequent drop in EPSP amplitude (in which amplitudes dropped to baseline levels) while the preps exposed to NF_1 alone continued to demonstrate an increase in EPSP amplitudes. The data is consistent with our hypothesis in that preps in which PKC was inhibited were unable to sustain EPSP amplitude increases induced by NF_1, indicating that PKC plays a role in this effect over time.

Our data also indicated that although PKC plays a role in causing the effects of NF_1, it is not the only protein kinase that is responsible for NF_1 's effects. Preps exposed to NF_1 and staurosporine, a broad spectrum protein kinase inhibitor, demonstrated a greater decrease in amplitude than preps exposed to NF_1 and LY 333 531. The fact that nonspecific inhibition of protein kinases caused a greater decrease in EPSP amplitude than specific inhibition of PKC indicates that other protein kinases also play a key role in the effects of NF_1. This invites further research into the roles other protein kinases may play in the effects of NF_1, as well as research comparing the significance of said proteins in relation to NF_1 and DF_2.

Our study yielded similar results to previous research on NF_1 in crayfish neuromuscular preps, in that it indicates that NF_1 exposure leads to an increase in EPSP amplitude over time (Skerret et al, 1999). Our results are similar to those of previous investigations into the role of PKC in the effects of DF_2 in crayfish, in that researchers studying DF_2 also found that PKC played a role in causing a sustained increase in EPSP amplitude (Freidrich et al, 1998). Our findings are significant, however, in that they fill a gap in the literature, providing insight into the role of PKC and in causing the effects of NF_1, a topic on which we did not find any previous research.

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