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Inhibition of Calcineurin Decreases Low-Frequency Depression and Increases Long-Term Potentiation in Crayfish Superficial Extensor Muscle Cells

TINA CHEN, COLIN DOWDELL, and ARIEL NELSON
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Long-term potentiation (LTP) and low-frequency depression (LFD) are two major forms of synaptic plasticity that regulate the strength of neurotransmission. Activities of calcineurin, a Ca^{2+}/calmodulin-dependent phosphatase, have been found to be involved in the long-term plasticity at synapses. Studies have established that inhibition of calcineurin blocks the induction of LFD, but questions still remain for specific effects on LTP. To reinforce the effect of calcineurin inhibition on LFD as well as to explore its effect on LTP, we applied the calcineurin inhibitor FK-506 to crayfish neuromuscular junctions undergoing LTP and LFD. Electrical stimulations of 0.2 Hz and 2 Hz were applied through an extracellular microelectrode to trigger LFD and LTP, respectively. We used an intracellular microelectrode to record excitatory post-synaptic potentials (EPSPs) in the superficial extensor muscle cells. Our results showed that after FK-506 was applied, the normalized peak amplitudes of EPSPs decreased under LFD and increased under LTP, supporting our hypothesis that the inhibition of calcineurin would decrease LFD and increase LTP at the neuromuscular junctions of superficial extensor muscle cells.

INTRODUCTION

Recent research shows that synaptic plasticity modifies the efficiency of synaptic transmission through various forms, such as potentiation or depression. These forms are commonly regulated by the changes in phosphorylation of synaptic proteins (Silverman-Gavrila & Charlton, 2009).

Phosphorylated proteins and their substrates facilitate long-term potentiation (LTP) in neurotransmission. LTP, a long-lasting increase in synaptic strength following high-frequency stimulation, leads to an increase in excitatory post-synaptic potentials (EPSPs) amplitudes. In contrast, dephosphorylated proteins and their substrates facilitate low-frequency depression (LFD), a frequency-dependent phenomenon observed at glutamatergic synapses in invertebrates. A decrease in transmitter release and a long-lasting depression in the amplitude of EPSPs have been found present under LFD. Silverman-Gavrila et al. (2005) demonstrated that, at the crayfish leg extensor muscle, LFD occurred with stimulation at 0.2 Hz and developed with time constants of 4 and 105 minutes to reach more than 50% depression of transmitter release in 1 hour.

Protein kinases lead to phosphorylation, which occurs when phosphate groups bind to synaptic proteins, while protein phosphatases reverse the process. Previous study has found out that blockade of protein kinases accelerated the slow phase of LFD at the crayfish leg extensor muscle cell, but stimulation of kinases reduced depression. Moreover, blockade of protein phosphatases 1A/2A reversed the slow phase. Thus changes in neurotransmitter release might occur by alteration in the relative activities of protein kinases and phosphatases (Silverman-Gavrila et al. 2005).

Calcineurin, a protein phosphatase, might be activated by directly binding with Ca^{2+} or calmodulin. Silverman-Gavrila et al. (2013) claimed that a protease, calpain, could also activate calcineurin by cleaving the regulatory domain of calcineurin. Previous study showed that inhibition of calcineurin by intracellular pre-synaptic injection of autoinhibitory peptide in a crayfish leg extensor muscle cell inhibited LFD and resulted in facilitation of transmitter release. However, when the inhibitor was injected to the post-synaptic cell, it had no major effects; thus only the pre-synaptic activity of calcineurin is necessary to induce LFD (Silverman-Gavrila & Charlton, 2009). Another study on the effect of calcineurin inhibition on LTP found that during the application of calcineurin inhibitor FK-506, short tetanus in visual cortical slices of young rats, which had rarely induced LTP in the normal medium, became effective in inducing LTP (Funauchi et al. 1994). These results suggest that calcineurin plays a role in regulation of long-term synaptic plasticity.

While recent research has been done on the effect of calcineurin inhibition on LFD at crayfish leg extensor muscle cell, we were interested in investigating its effect on LTP at a different crayfish muscle cell, which is the superficial extensor muscle cell in the crayfish tail. Furthermore, previous study stated that calpain-mediated

cleavage of calcineurin could be attributed to neurodegenerative diseases, such as Alzheimer's and Huntington's diseases (Wu et al. 2004). Therefore, to explore new medical treatments for these diseases, it is worthwhile to investigate specific effects of calcineurin inhibition on synaptic plasticity.

We sought to reinforce the previous findings about LFD as well as to explore LTP relating to calcineurin inhibition in the pre-synaptic crayfish neuromuscular junctions. Through an extracellular electrode, we stimulated the crayfish superficial extensor muscle nerve at different frequencies to trigger LTP and LFD and applied FK-506 to the crayfish Ringer's solution. EPSPs were measured by an intracellular electrode. Our results showed that after the application of FK-506, the normalized peak amplitudes of EPSPs decreased under LFD and increased under LTP, supporting our hypothesis that calcineurin inhibition would decrease LFD and increase LTP at the neuromuscular junctions of superficial extensor muscle cells.

MATERIALS AND METHODS

Preparation of Crayfish Tail

To begin the experiment, a crayfish (*Procamburus clarkii*) was immobilized in ice at least 15 minutes. Then the crayfish tail was cut off at the base and extracted from the rest of the body. Using a scissor blade, an incision was made on both sides of the crayfish tail, cutting closest to the ventral surface all the way to the posterior end. Then the ventral surface and the muscle mass were pushed out from the tail using the thumb to expose the dorsal exoskeleton and extensor muscle of the crayfish. The tail was pinned down to the dissection dish with three pins and submerged in crayfish Ringer's solution. This solution has a pH of 7.4 (Lindgren), and a composition consisting of 196 mM NaCl, 5.4 mM KCl, 13.5 mM $CaCl_2$, and 2.6 mM $MgCl_2$ (Mezochow, 2010).

Exogenous Drug Application

Under experimental conditions of LTP and LFD, a 100 ml solution containing FK-506 replaced the crayfish Ringer's solution to inhibit calcineurin. To create our stock solution, 80 µl of 50 mM FK-506 dissolved in DMSO was added to 100 ml of Ringer's solution and the final concentration of our FK-506 stock solution was 40 µM. We chose the final concentration of FK-506 based on the concentration used in the study by Silverman-Gavrila and Charlton (2009). For the negative DMSO control experiment, we used 100 ml of Ringer's solution with 80 µl of DMSO in order to be consistent with the volume of FK-506 applied.

Microelectrodes Preparation

We used a WPI PUL-1 Micropippette Puller to heat up a glass tube (1.2 mm in diameter —small enough to penetrate individual muscle cells causing minimal damage) until it was pulled into two fine tipped micropipettes. For making an intracellular microelectrode, we used a fine syringe to fill one electrode and an electrode holder with 3.0 M KCl, making sure there were no air bubbles. We tested the condition of the intracellular microelectrode by measuring its resistance, which was between 5 and 10MΩ. An extracellular electrode was formed similarly to the intracellular electrode but the tip was filed using Emory paper to create a wider tip diameter for the suction of the crayfish nerve. We backfilled the extracellular microelectrode with Ringer's solution and used a syringe to suck the crayfish nerve into the microelectrode. Different frequencies of electrical stimulations were also applied through the extracellular microelectrode.

Intracellular recording Technique and Electrical Stimulation Application

With a micromanipulator, the intracellular microelectrode was maneuvered into the crayfish superficial extensor muscle cells allowing EPSPs to be recorded using the computer program SCOPE (Mac v.4.1.1). EPSPs were detected by the MacLab Bridge Amp and digitized by a PowerLab data acquisition system (AD instruments, Model ML846). The crayfish motor nerve was pulled into the extracellular suction electrode, which was connected to an SD9 Stimulator (Astro-Med Inc., Model SD9K), and was stimulated at 0.2 Hz and 2 Hz to induce LFD and LTP, respectively. To record baseline EPSP peak amplitudes, which were then compared to the amplitudes under conditions of DMSO, LFD, LTP, LFD with inhibitor and LTP with inhibitor to observe the changes in EPSPs, we stimulated the nerve at 0.02 Hz based on the frequency used in Silverman-Gavrila and Charlton's study (2005). To allow for better conditions in which experimental data were to be collected, the lights from our microscope were turned off. We were extremely careful and meticulous in our set-up and before starting each experiment, we tested the condition of the intracellular microelectrode.

Data Analysis

Through Scope for Mac v.4.1.1, we measured peak amplitude interpreted as the difference between pre-EPSP events and the peak of the rising phase of the EPSP. Thereafter, we normalized our data to the first EPSP peak amplitude in each experiment and created analyses based off the rate of change of the normalized EPSP peak

amplitudes during DMSO control, LFD and LTP experiments.

RESULTS

To study the effect of calcineurin inhibition on LFD and LTP, we used the immunosuppressive drug FK-506, which is known to be a specific and potent calcineurin inhibitor (Da-Yu et al. 2006), and measured the peak amplitudes of EPSPs through intracellular recordings. We normalized the EPSP peak amplitudes to the first EPSP peak amplitude in each experiment and analyzed the rate of change in the normalized amplitudes under conditions of DMSO, LFD, LTP, LFD with inhibitor and LTP with inhibitor. During each experiment, we changed the crayfish saline every 15 minutes to maintain a normal function at the crayfish neuromuscular junctions.

LFD depresses synaptic transmission

We conducted the first experiment to evaluate the effects of LFD on synaptic transmission presented by Silverman-Gavrila et al. (2005). To record baseline EPSP amplitudes, we first applied 0.02 Hz stimulation to the crayfish nerve by stimulating it every 50 seconds for around 10 minutes. In Figure 1, the normalized peak amplitudes showed that EPSPs amplitudes remained relatively stable within the range of 100 and 140 percent. We then increased the frequency to 0.2 Hz with 5-second intervals for about 30 minutes, LFD occurred following a plummet of EPSP amplitudes from 100 to around 20 percent, shown in Figure 1. Instead of continuing on to the full hour of testing, we stopped around 40 minutes because EPSP amplitude had already exceeded a 50 percent decline, as observed in the previous study by Silverman-Gavrila et al. (2005). From there, we decreased the frequency back to baseline (0.02 Hz) and it remained for the rest of the experiment until a little over an hour. Figure 1 shows that the normalized EPSP peak amplitudes did not return back to 100 percent and LFD was still in effect through the rest of the experiment.

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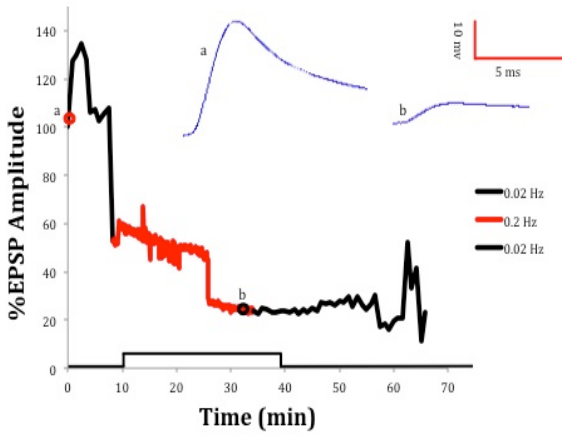

Figure 1. LFD has a long-lasting effect on synaptic transmission. The decline in normalized EPSP peak amplitudes was not halted after changing the stimulation frequency back to baseline (0.02 Hz). Representative EPSP recordings are shown at the beginning of the experiment during non-depressing baseline (t=0) and at LFD (t=33).

DMSO increases synaptic transmission after 5 minutes

The DMSO experiment was conducted in the solution consisting of 100 ml of saline with 80 µl of DMSO. We stimulated the nerve at baseline of 0.02 Hz throughout the entire experiment. As shown in Figure 2, during the first 15 minutes of the experiment, we observed the EPSP amplitudes to be relatively stable. However, after 15 minutes, there was an about 200 percent drastic increase in EPSP amplitude.

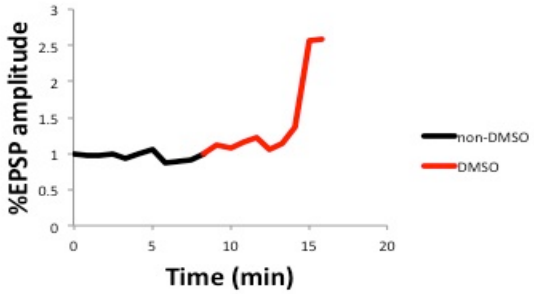

Figure 2. DMSO has an added effect on EPSP amplitude after minute 5. The data above is taken from two separate days—on one day, the non-DMSO Ringer solution was stimulated at a non-depressing baseline of 0.02 Hz and the next day, the DMSO-infused solution was stimulated at the same non-depressing baseline. DMSO increased EPSP amplitude at 5 minutes and thereafter, EPSP stabilized at higher amplitudes.

Calcineurin inhibition reduces LFD

Through exogenously introducing FK-506, calcineurin was inhibited locally at the presynaptic cell, since Silverman-Gavrila and Charlton (2009) noted that the inhibition of calcineurin had no effect on the postsynaptic cell. With respect to our results from the first and second experiments, we limited LFD to 3 minutes in order to avoid interference from the long-lasting effects of LFD and DMSO.

To establish a consistent EPSP reading, we recorded baseline (0.02 Hz) for 5 min. and then induced LFD at 0.2 Hz for 2 minute. To return the EPSP peak amplitudes to initial baseline recordings, we then returned the frequency back to 0.02 Hz and simulated the nerve for 35 minutes. As shown in Figure 3, the normalized EPSP peak amplitudes decreased continuously since the experiment began and suddenly increased by a about 30 percent after 30 minutes. This shows that the EPSP peak amplitudes did not start to increase until 23 minutes after the second baseline stimulation and did not return back to the initial baseline peak amplitude.

At the last 5 minutes of the experiment, we replaced the Ringer's solution with stock solution with FK-506 and induced LFD. By measuring the maximum slope of each EPSP phase, we observed that during LFD induction with Ringer's solution, normalized EPSP peaks decreased by 2.7 percent/min while during LFD induction with stock solution, normalized EPSP peaks decreased by 1.2 percent/min. Thus Figure 3 demonstrates that after FK-506 was applied, the peak amplitudes of EPSP did not decreased as much as it did in Ringer's solution.

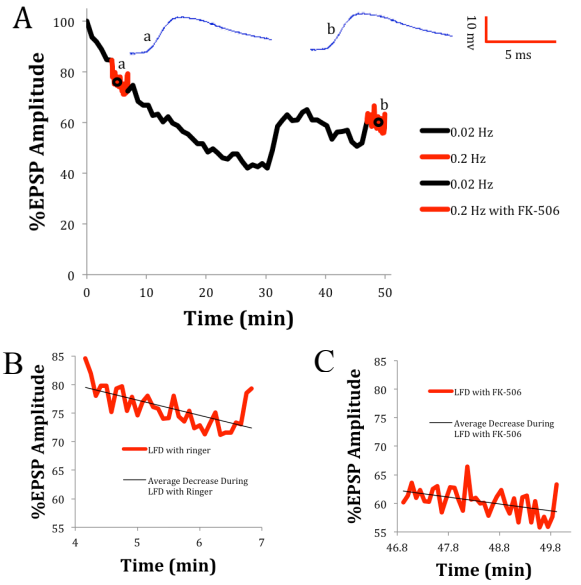

Figure 3. Calcineurin inhibition facilitates synaptic transmission. A) During baseline recordings for the second time, long-term LFD effects could be seen until around the 30-minute mark. Non-depressing baseline was observed over nearly 40 minutes in order to prevent interference from the long-lasting effects of LFD. LFD in the presence of FK-506 began stimulation at 0.2 Hz in a non-depressed environment. Representative EPSP recordings are shown halfway through LFD in Ringer solution (t=6) and at LFD (t=45). B) The average rate of depression during LFD in ringer solution was at 2.7 percent/min. Averages of this section of data indicated that LFD was present. C) The average rate of depression during LFD in ringer solution was at 1.2 percent/min. Averages of this section of data indicated that LFD was inhibited, and facilitation occurred instead.

Calcineurin Inhibition Potentiates LTP to a greater degree

In order to maintain comparable results and to avoid interference from DMSO, we limited LTP to 3 minutes as well. Similar to our previous experiment of LFD with inhibitor, we measured the baseline for 5 minutes and then induced LTP at 2 Hz for 15 seconds (to keep consistent, we took 30 measurements, regardless of interval). Figure 4 shows that LTP did not seem to cause any noticeable increases in the normalized EPSP peak amplitudes after its induction. We then decreased the frequency to 0.02 Hz and simulated the nerve at baseline for 30 minutes in order to observe any possible long-term effects of LTP. In Figure 4, the normalized peak amplitudes showed a continual drop from the beginning of the experiment and at 20 minutes of the experiment, there was a bump in the EPSP amplitudes.

At the last 50-second period of the experiment, stock solution with FK-506 replaced the Ringer solution and LTP was induced. The maximum slope of each EPSP phase illustrates that during LTP induction with Ringer's solution, normalized EPSP peaks increased by 16 percent/min while during LTP induction with stock solution, normalized EPSP peaks increased by 82 percent/min. As shown in Figure 4, after FK-506 was applied, the normalized EPSP peak amplitudes increased above the initial baseline amplitude.

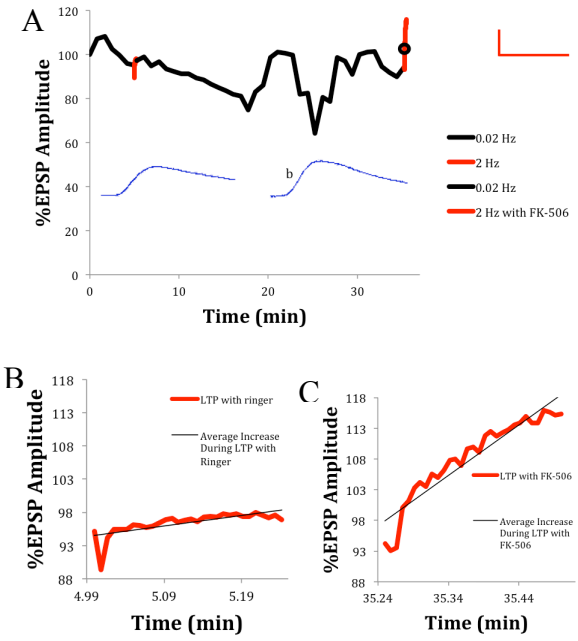

Figure 4. Calcineurin inhibition potentiates synaptic transmission in LTP to a greater degree than naturally induced LTP. A) LTP did not seem to have any noticeable long-term effects in baseline recordings. Representative EPSP recordings are shown halfway through LFD in Ringer solution (t=6) and at LFD (t=37). The rise in potentiation during LTP in stock solution is larger than the rise in potentiation during LTP in Ringer solution. B) The average rate of potentiation during LTP in ringer solution was at 16 percent/minute. Averages of this section of data indicated that LTP was potentiated.

C) The average rate of potentiation during LTP in stock solution with FK-506 was at 82 percent/minute. Averages of this section of data indicated that LFD was potentiated to a greater degree.

DISCUSSION

We hypothesized that the application of FK-506 would cause lower EPSP amplitudes under LFD and higher EPSP amplitudes under LTP in the pre-synaptic crayfish superficial extensor muscle cell. To study the effect of calcineurin inhibition on LFD and LTP, we applied the calcineurin inhibitor FK-506 to crayfish Ringer's solution under LFD and LTP, which were triggered respectively at 0.2 and 2 Hz through an extracellular electrode. We used an intracellular microelectrode to record EPSPs in the superficial extensor muscle cells. Our results showed that after FK-506 was applied, the normalized peak amplitudes of EPSPs decreased under LFD and increased under LTP, supporting our hypothesis.

As shown in Figure 1, the amplitude of EPSPs showed a deep reduction from 100% to about 30% over 60 min since LFD was activated. This supports the findings in the previous study by Silverman-Garvila & Charlton (2009). According to Bruner and Kennedy (1970), at frequencies close to LFD (0.02 Hz) a sharp decline in the amplitude occurred due to hypothesized electrical changes in presynaptic terminals. We argue that the remarkable decline in the EPSP peak amplitudes is due to the long-lasting effects of LFD because when the experiment lasted for an extensive amount of time, there was a decrease in overall EPSP amplitude, as the cell was exhausted from stimulus (Figure 1).

The results of the DMSO negative control group remain inconclusive. Figure 2 shows that after 15 minutes of the experiment, there was an unexpected rise in the EPSP amplitudes. However, in previous experiments involving immunosuppressant inhibitors such as FK-506, DMSO was primarily used as a solvent and assumed to have no added effect (Rusnak & Mertz, 2000; Silverman-Gavrila et al., 2005; Silverman-Gavrila et al., 2013; Silverman-Gavrila & Charlton, 2009). Thus we are not able to attribute the results of the DMSO control experiment to previous studies.

As seen in Figure 3, after FK-506 was applied, we observed that the amplitude of EPSPs under LFD decreased less than without FK-506. We propose that this change is due to facilitation occurring instead of depression when calcineurin is inhibited because with the inhibition of calcineurin, there was a reduction of phosphorylation of synaptic proteins, resulting in an increase in neurotransmitter release. In Silverman and Charlton's study (2005), stimulation at 0.2 Hz under calcineurin induction caused facilitation rather than depression. Similarly, through our data, we recognized facilitation as having less depressive effects than induced LFD.

When LTP was conducted with FK-506, there was an increase in the normalized EPSP peak amplitude (Figure 4). This result is consistent with Beaumont's study (2005) in which stimulation at 2 Hz under calcineurin induction potentiated normalized EPSP amplitudes to a greater degree than normal LTP. Thus we attribute the results of the LTP experiment to the increased rate of neurotransmitter facilitation.

Although our results sufficiently support the hypothesis, there are still some factors that contributed to errors and led to inaccuracy of data in the study. For example, we changed the crayfish Ringer's solution every 15 min during each experiment and it is possible that the change of solution disturbed the intracellular recording of EPSPs; thus, resulted in unintentional deviations within the data. Also, the effect of DMSO on synaptic transmission was not fully ruled out in our study because DMSO started to show an effect 15 min after it was applied to the solution. Even though we kept the crayfish tail in FK-506 solution for less than 15 min, we are not confident enough to argue that DMSO has no effect on neurotransmission in the study.

Since we only did one trial of each experiment, the EPSPs recorded may not accurately reflect the average change of amplitudes under those conditions. Therefore, to obtain more accurate results, we should complete at least three trials for each condition in future studies. Another limitation of our study is the length of FK-506 application. Because the DMSO control experiment suggested that DMSO showed an effect 15 min after it was applied, we only left the crayfish tail in FK-506 solution for about 3 min, which is much shorter than the amount of time used in previous study. If we ruled out the effect of DMSO, we would apply the inhibitor for a longer period of time to examine whether EPSPs show a similar change.

Moreover, previous study showed that the activation of the calcium-dependent protease calpain facilitates constitutive activities of calcineurin (Silverman-Gavrila et al. 2013). Therefore, future research could be done on the relationship between calpain and calcineurin. Inhibitors of calpain could be applied to test the effects of calpain activity on calcineurin functions. Because calpain-mediated activation of calcineurin A triggers excitotoxic neurodegeneration, which might cause neuronal death in the brain and thus lead to neurodegenerative diseases (Wu et al. 2004), it is worthwhile to further investigate how to prevent neuronal damage and death through the use of calpain inhibitors. Furthermore, we could study the effect of calcineurin facilitation on LFD and LTP at neuromuscular junctions by applying an activator of calcineurin.

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