Decreased calcium concentration increases magnitude and time course of facilitation but decreases time course of depression following long-term high frequency stimuli.

AUSTIN GRAVES, HARIS IQBAL, and JESSICA PIERSON
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT
Calcium is a ubiquitous second messenger in neurotransmitter release pathways, but the specific mechanisms of its regulation of synaptic plasticity are still a topic of debate. Our study further clarifies the effects of low calcium concentration on the magnitude and time course of initial synaptic facilitation and subsequent depression resulting from long-term high frequency stimulation at the crayfish neuromuscular junction. Our results contradict previous studies that suggest increased levels of pre-synaptic calcium concentration result in increased magnitude and time course of facilitation. These contradictory results may implicate additional roles for calcium, not only as a second messenger, but also as a buffer ion used to shield the surface charge of cells, thus maintaining a high neuronal threshold. Our results agree with the vesicle depletion hypothesis, that lower levels of calcium leads to a decreased time course of depression.

INTRODUCTION
Calcium is the most essential mineral in our body and plays an important role in the processes of synaptic transmission in the nervous system. Depolarization of pre-synaptic axon terminals opens voltage-gated calcium channels, increasing intracellular calcium concentration and releasing neurotransmitter (NT). While the general role of calcium as a second-messenger in NT release is clearly understood (Atluri and Regehr, 1996), much controversy exists regarding the mechanisms of calcium’s regulation of synaptic plasticity.

Atluri and Regehr (1996) found that increased pre-synaptic residual free calcium concentration increased facilitation and extended its time course through transmitter-release dependent mechanisms. Stevens and Tsujimoto (1995) suggest that depression is also linked to transmitter release-dependent mechanisms regulated by pre-synaptic residual free calcium concentration. Brody and Yue (2000) contradict these findings and suggest that the amplitude and time course of depression are independent of pre-synaptic residual free calcium concentration. They attribute their results to action potentials’ failure to effectively propagate down the axon, thus reducing pre-synaptic depolarization and subsequent NT release (Brody and Yue, 2000).

Our research examines the effect of external calcium concentration on facilitation and depression triggered by long-term high frequency stimulation at the crayfish neuromuscular junction. The post-synaptic response to stimulation was biphasic; we observed initial facilitation that quickly decayed as synaptic depression set in. We found that decreased calcium concentration increased the amplitude of initial facilitation, increased the facilitation time course, and decreased the time course of depression. Calcium concentration did not affect the magnitude of synaptic depression.

METHODS AND MATERIALS
Organisms and Dissection Technique
Experiments were performed using adult crayfish, *Procambarus clarkii*, obtained from the Carolina Biological Supply Company. We cold anaesthetized the crayfish and exposed the lateral deep extensor muscle and its innervating motor nerve. The dissection is fully outlined in the “Crawdad Lab Manual for Neurophysiology” (Wyttenbach et al, 2002).

Experimental Solutions
We used a Ringer’s solution of pH 7.4 comprised of 205 mM NaCl, 5.4 mM KCl, 2.6 mM MgCl\(_2\), 2.3 mM NaHCO\(_3\), and 1.0 mM Tris buffer. We varied the calcium concentration in our three experimental conditions, using 2.5, 5.0 and 10 mM CaCl\(_2\). Solutions with calcium concentrations above 10 mM were not used, as we found that they generated calcium action potentials that interfered with the accurate interpretation of the data. The solution was changed every 15 minutes to increase the longevity of the preparation.

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Electrophysiology and Measuring Facilitation and Depression

Standard intra and extra-cellular recording techniques were used. Recording electrodes had 0.4 μm diameter tips with ~8 MΩ resistance and were filled with 3.0 M KCl, (PUL-1 apparatus, World Precision Instruments). Suction electrodes had 10 μm diameter tips and were filled with Ringer’s (Flaming/Brown Micropipette Puller, Sutter Instruments).

Resting membrane potentials were 60-80 mV. We elicited synaptic depression by stimulating the nerve at 20 Hz for ~ 2 minutes (Grass SD9 stimulator). EPSPs were measured in the muscle every five seconds (MacLab/4 converter, Analog Digital Instruments). The initial EPSP amplitude (time=0) was defined as the baseline level (100%). We normalized subsequent EPSPs by dividing their amplitude by the baseline. Values above 100% indicate synaptic facilitation, while sub-baseline values represent depression. The time course of facilitation was defined as the elapsed time until the maximal level of facilitation was reached. The time course of synaptic depression was measured as the elapsed time from when mean percent EPSP values first dropped below 100% to when percent mean EPSP values decayed to 25% of the baseline.

RESULTS

High frequency stimulation yielded two distinct time courses at the crayfish neuromuscular junction, one for initial facilitation and another for eventual depression. Both of these time courses appear to have been affected by calcium concentration, as is the magnitude of initial facilitation. Data for mean EPSP amplitude relative to baseline values for 10, 5, and 2.5 mM calcium are presented in Figure 1.

Examination of the magnitude and time course of facilitation suggests that low calcium (2.5 mM) yields the greatest amount of initial facilitation (224% of baseline EPSP values, 15 seconds, n=2). 5 mM calcium elicits moderate initial facilitation (152% of baseline values at 15 seconds, n=3). The highest level of calcium (10 mM) yields small levels of initial facilitation (113% of baseline, 5 seconds, n=3). The lowest level of calcium corresponds to a significantly decreased time course of facilitation.

The second time course (depression) is also related to calcium concentration. Synapses under the low calcium condition (2.5 mM) began to depress after 55 seconds of high frequency stimulation, decaying to 25% of baseline EPSP values after 115 seconds (n=2). The mean time course of depression in 2.5 mM calcium concentration is 60 seconds.

Synapses in 5.0 mM calcium first depressed following 45 seconds of high frequency stimulation, falling to 25% of baseline after 110 seconds (n=3). This time course was 65 seconds. There is no significant difference between the time courses of these two experimental conditions. However, synapses under high calcium conditions (10 mM, n=3) began to depress faster, after 15 seconds, and decayed to 25% of baseline EPSP values significantly more rapidly (85 seconds) than either 5 or 2.5 mM calcium conditions. The statistically significant time course of depression for this condition is 75 seconds. EPSPs were never completely abolished in any experimental condition; all three depressed to a value of ~15% of baseline EPSPs after two minutes of high frequency stimuli. Calcium concentration did not affect this magnitude of depression. Synaptic depression was not measured beyond two minutes.

DISCUSSION

We examined the biphasic post-synaptic response to long-term high frequency stimulation. Our results suggest four major conclusions regarding the effects of low calcium concentration on synaptic plasticity at the crayfish neuromuscular junction (nmj): low calcium (1) increases the magnitude of initial facilitation, (2) increases the time course of
facilitation, (3) does not affect the magnitude of depression, (4) and decreases the time course of depression.

(1) Our finding that decreased calcium concentration increases facilitation (Fig. 1) contradicts the accepted hypothesis that calcium’s role is primarily as a second messenger in neurotransmitter (NT) release. If this were the case, higher levels of calcium would have elicited an increased magnitude of facilitation. Our results implicate a potential additional role for calcium in the regulatory mechanisms of synaptic plasticity. Calcium acts as a buffer by shielding the surface charges of cells throughout the nervous system, rendering them less depolarizable (Personal correspondence with Dr. Clark A. Lindgren, 2003). Decreasing the calcium concentration reduces this stabilizing effect by decreasing the voltage gradient across the membrane. Thus, low extracellular calcium increases the probability that sufficient voltage gated calcium channels will open, leading to a depolarization of the pre-synapse. This will increase NT release. The calcium-buffering hypothesis explains our findings that decreased calcium concentration increases the magnitude of initial facilitation and poses an intriguing additional role for calcium in the mechanisms of NT release. We could test this assertion by decreasing the pH, which would in turn lower the effects of the voltage gradient and alter the magnitude of facilitation.

(2) At low concentrations of calcium we observed an increase in the time course of facilitation (Fig. 1). These results can also be attributed to the calcium-buffering hypothesis. We have established a potential link between low calcium and increased magnitude of facilitation, caused by increased quanta in the cleft. We may further hypothesize that greater concentrations of released quanta would increase the time course of facilitation, as it would take longer to break down and deactivate these high concentrations of NT. At lower levels of calcium, facilitation and subsequent quantal release are both increased, thus extending the elapsed time of increased NT release and facilitation.

(3) We found that the magnitude of synaptic depression was independent of calcium concentration for the first two minutes of high frequency stimulation (Fig. 1). The traces for all three of our experimental conditions (10, 5, and 2.5 mM calcium) approached a static value for depression of ~15% of baseline EPSP amplitude. This finding concurs with the prevailing hypothesis that the limit to the maximum potential magnitude of depression is ~10-fold (Nicholls, 2001).

(4) Low calcium concentrations decreased the time course for synaptic depression (Fig. 1), a finding supported by Dittman et al. (2000). The widely accepted vesicle depletion mechanism explains these results. Lower levels of calcium increase initial facilitation, thus increasing initial quantal release. This high rate of vesicle exocytosis cannot be maintained for long, however. As the vesicle pool begins to run out, the synapse begins to depress. The time course of depression is more rapid in low levels of calcium because of the heightened initial facilitation and corresponding increased rate of vesicle exocytosis. This leads to vesicle depletion and an increase in the rate of depression, thus reducing the depression time course.

The regulatory mechanisms of calcium examined in this study elucidate the workings of synaptic plasticity as a whole. These mechanisms are all intimately related to key processes in learning and memory. The potential roles of calcium in these regulatory mechanisms have not been adequately defined, as evidenced by our contradictory results. Our data did not refute the possibility that changes in pre-synaptic calcium may be due to failure of action potentials to effectively propagate down the axon; further proposing that facilitation and depression are calcium independent (Brody and Yue, 2000). Further research must be conducted regarding the potential involvement and exact mechanisms of pre-synaptic calcium on the time course and magnitude of facilitation and depression to completely elucidate the mechanisms behind learning and memory.

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REFERENCES


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