Pioneering Neuroscience, 2000, **1**, 41-47

Bapta-AM Has No Significant Effects On Synaptic Plasticity At The Crayfish Lateral Extensor Neuromuscular Junction

AMY BAILEY, MEGAN SAMUELSON, and LAUREL STEINMETZ
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Short-term synaptic plasticity (more specifically, facilitation and depression) has been linked to long-term plasticity, which is involved in memory formation. Facilitation and depression are opposing aspects of plasticity that work dynamically through calcium-mediated mechanisms. We tested the effects of reduced intracellular calcium levels on synaptic plasticity using paired-pulse stimulation of crayfish lateral extensor muscles and the fast calcium chelator BAPTA-AM (bis-(aminophenoxy)ethane-tetraacetic acid, membrane permeant acetomethoxy ester form). While we expected facilitation, we observed only depression in each of our treatments. The control of DMSO (dimethyl sulfoxide) in saline had significantly greater EJP amplitudes than either the saline control or the BAPTA-AM treatment, which had similar EJP amplitudes. We found that BAPTA had no significant effects on synaptic plasticity at either 25 or 35 msec time delay between paired-pulse intervals, though our control treatments of either saline or DMSO in saline were significantly different for each experiment. Our results did not support or refute previous studies on the role of calcium in synaptic plasticity.

INTRODUCTION

Synaptic plasticity is thought to be responsible for memory formation. Xie *et al.* (1996) found that neurons expressing long-term potentiation are involved in learning and memory. Furthermore, some studies have shown that induction of long-term potentiation is influenced by previous synaptic activity; more specifically, short-term facilitation was found to have an effect on a neuron's ability to exhibit long-term potentiation. The short-term facilitation is thought to act as a "primer" for the longer potentiation experienced by the neuron later in time (Hsu *et al.*, 1999). In addition to facilitation, depression is another form of synaptic plasticity that has been implicated in memory formation and habituation. For example, synaptic depression appears to be involved in the decrease of the crayfish escape response (i.e. tail flick) when a repeated stimulus is given (Zucker, 1989). Therefore, the mechanisms underlying short-term synaptic plasticity are key to understanding more complex neuronal processes.

Currently, there are several explanations regarding synaptic depression. Depression is often correlated with the decrease of released neurotransmitter, due to a number of possibilities including depletion of presynaptic vesicles, reduction of intracellular calcium, or inhibition of receptors (Zucker,

1989 and Zucker *et al.*, 1999). However, a decrease in released neurotransmitter cannot completely explain depression. For example, Wang and Kelly (1997) used microinjection of BAPTA and found that a postsynaptic calcium-dependent pathway may be involved in synaptic depression.

Facilitation is observed only when depression is masked. Research regarding the mechanism of short-term facilitation is divided, but most researchers believe that the residual effects of calcium ultimately cause facilitation. While the current views are conflicting, the residual effects of calcium could be caused by either free (Kamiya and Zucker, 1994) or bound residual calcium in the presynaptic cell, or the lasting cellular effects from the initial calcium influx (Blundon *et al.*, 1993). Calcium chelators are often used to test which of these two mechanisms (elevated calcium levels or lasting effects) are responsible for facilitation. Van der Kloot and Molgo (1993) found that BAPTA-AM reduced synaptic facilitation in frogs when time delays larger than 30 msec were used.

BAPTA is a fast calcium chelator (Ouanounou, 1999), and its affinity for calcium (100nM) brings the intracellular calcium levels down to non-stimulated concentrations (Tymianski *et al.*, 1994). Hence, it may have adverse effects on singular EJP amplitude as well as synaptic facilitation. Because of these properties, we examined BAPTA-AM's effects on short-term synaptic plasticity and EJP amplitude. Specifically, we treated crayfish neuromuscular junctions with membrane-permeable BAPTA-AM to determine if this treatment reduces synaptic facilitation. We attempted to determine whether synaptic facilitation was due to calcium remaining in the cell as opposed to the after-effects of calcium that is no longer present in the cell. However, due to BAPTA's affinity for calcium, we could not distinguish between bound and free calcium within the cell. We anticipated that this chelator would decrease synaptic facilitation, but then observed no synaptic facilitation in control treatments or those treated with BAPTA-AM.

MATERIALS AND METHODS

Preparations.

We used ice-anesthetized crayfish in order to examine facilitation in the neuromuscular junctions of the lateral extensor muscles. The crayfish were dissected to isolate the superficial extensor muscle as described by Stephens (1996). Each preparation was incubated for one hour with one of the following three treatments: (1) crayfish saline, (2) 10 µL of DMSO in 10ml crayfish saline (0.001 percent by volume), or (3) a solution of BAPTA-AM in DMSO (10mM), diluted with crayfish saline to a final concentration of 10µM. The preparations were incubated with cell permeant BAPTA-AM for one hour to allow nonspecific cellular esterases to cleave the acetomethoxy group from the internalized BAPTA-AM. Removal of the AM group both traps and activates the BAPTA. Extracellular calcium levels remained constant because uncleaved BAPTA-AM cannot chelate calcium. Following incubation, each treatment was washed with crayfish saline for 30 minutes and then bathed in this solution for the remainder of the experiment (Lindgren *et al.*, 1997).

Paired-pulse measurements.

Our experimental data was obtained using paired-pulse stimulation of the caudal neuron innervating the lateral extensor muscles in the tail of the crayfish. The electrical pulse was generated by a Grass

stimulator, and the subsequent excitatory junction potentials (EJPs) were measured with a microelectrode filled with 3M KCl; we then used MacIntosh *Scope* to record the data. For each of the treatments, we used a frequency of 0.5 pps and a delay between paired pulses of either 25 or 35 msec.

Statistical Analysis.

For all conditions used, the mean of initial EJP amplitude was calculated. Additionally, we calculated the mean of the ratio $[(EJP_2-EJP_1)/EJP_1]$ between paired-pulse amplitudes for all treatments at both 25 and 35 msec delays. From this ratio, depression gives a negative number and facilitation gives a positive number. Significance of each mean was determined using ±2SEM. Graphs were made using Microsoft Excel.

RESULTS

Saline controls with and without DMSO showed paradoxical results.

At a time delay of 25 msec between paired-pulse stimulation, our control of saline showed a significantly lower amount of synaptic depression than that of our saline/DMSO control. However, we observed that at a 35 msec time delay, our saline control showed a significantly greater amount of depression than that of the saline/DMSO control (Fig. 1). The inconsistencies between controls continue with the fact that initial EJP amplitude of the saline control was 40 percent of the saline/DMSO control's amplitude (Fig. 2). While there is a remote possibility that the DMSO is responsible for these differences, Cummings *et al.* (1996) found that a 0.25-0.5 percent solution of DMSO in saline, which is much greater than the concentration (0.001 percent by volume) used in our experiment, has no significant effects on synaptic plasticity. Therefore, our results here may be due to differences in crayfish.

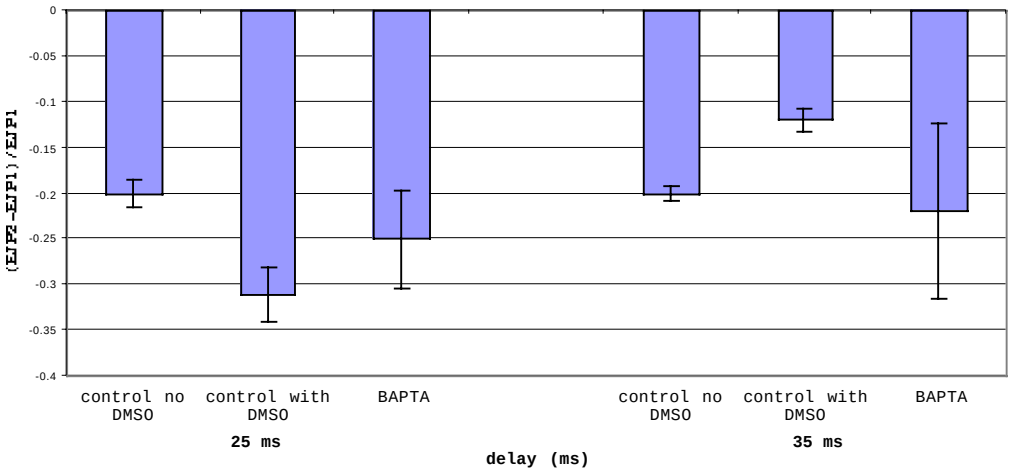
Initial EJP amplitude for BAPTA-AM treatments were significantly lower than the saline/DMSO control, but similar to those of the saline control.

The EJP amplitude of BAPTA-AM-treated preparations was 40 percent of the saline/DMSO treatment's amplitude (Fig. 2). This observation might be explained by BAPTA-AM's ability to chelate calcium quickly, which would cause lower initial EJPs when compared to a treatment without a chelating agent. However, the saline control without DMSO showed very similar initial EJP amplitudes to the BAPTA-AM-treated amplitudes (Fig. 2).

BAPTA-AM-treated preparations at 25 msec delay between paired-pulse stimulation are not significantly different from either control.

Compared to the saline control, the BAPTA-AM treatment showed greater depression, though it was not significantly different (Fig. 1).

This result is consistent with our hypothesis that BAPTA-AM decreases facilitation. However, this result is inconsistent with studies that have found that a decrease in calcium in turn decreases depression. Our observation that there was less depression in the BAPTA-AM-treated preparations than that of the saline/DMSO control, though also insignificant (Fig. 1), concurs with these previous studies. This result may also be due to the larger initial EJPs observed for the saline/DMSO control. Through research using EGTA-AM, Parker (2000) found that larger EJPs are linked with a larger amount of neurotransmitter release; therefore, the saline/DMSO treatment may have depleted its neurotransmitter storage during the first stimulation, thus leading to a smaller EJP_2.



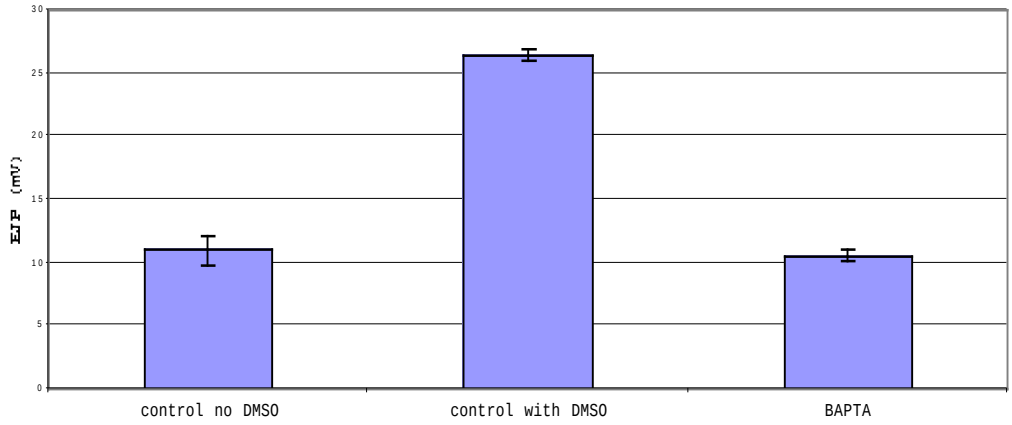


Figure 1 *(top).* All treatments showed synaptic depression rather than facilitation. Significant differences occurred only between saline and saline/DMSO controls at both 25 and 35 msec. Treatments with BAPTA showed no significant differences. Average normalized synaptic depression [$(EJP_2-EJP_1)/EJP_1$] was measured for crayfish treated with either BAPTA-AM, a saline/DMSO solution, or saline for 1 hr and washed in saline for 30 min. Crayfish were stimulated with a twin pulse (frequency 0.5 pps and delay of 25 or 35 msec). n = 10 trials for all treatments but the 35 msec BAPTA-AM treatment where n = 20. Error bars represent ±2SEM.

Figure 2 *(bottom).* EJP amplitudes were measured for each treatment. BAPTA-AM-treated preparations and the saline control had similar EJP amplitudes and were 40 percent of the saline/DMSO control's amplitudes. Crayfish preparations were treated for one hour with BAPTA-AM, a saline/DMSO solution, or saline for 1 hr and washed in saline for 30 min. n = 10 trials for each treatment. Error bars represent ±2SEM.

A 35 msec delay between paired-pulse stimulation causes greater depression in BAPTA-AM-treated preparations than either saline or saline/DMSO controls.

While no facilitation was observed in any of our treatments, the BAPTA-AM-treated preparations showed the most depression, though this depression was not significant (Fig. 1). An insignificant result may be due to the fact that the two separate crayfish preparations using BAPTA-AM differed vastly in EJP amplitude ratios (normalized using $[(EJP_2-EJP_1)/EJP_1]$). We used only one crayfish preparation for the other two treatments, which is reflected in the smaller deviation from the mean in these preparations.

DISCUSSION

Because of our insignificant results, we are unable to conclude whether or not BAPTA-AM decreases synaptic facilitation or depression. Regarding singular EJPs, BAPTA-AM-treated preparations had significantly smaller amplitudes than the saline/DMSO control, which might be expected because of BAPTA-AM's fast chelating abilities. However, the two controls also differed significantly in their mean EJP amplitudes, with the saline control's amplitudes being much lower than that of the saline/DMSO treatment's amplitudes. Further, the saline control's mean EJP amplitude was similar to that of the BAPTA-AM-treated preparations' amplitudes. Analysis of our paired-pulse stimulation trials showed that BAPTA-AM increased depression at the 35 msec delay, but its mean EJP ratio was between the two controls at the 25 msec delay (Fig. 1). The 35 msec delay results follow our hypothesis, but the 25msec delay results negate it. Furthermore, we cannot accurately compare the BAPTA-AM treatment's results to our two controls (saline and saline/DMSO) because the controls themselves show opposite results. In fact, the controls were the only treatments showing significant differences. Previous studies have shown that DMSO at a very low concentration has no effect on synaptic plasticity (Cummings *et al.*, 1996), and low calcium levels cause both decreased facilitation and depression (Zucker, 1989). Therefore, we believe that the discrepancies in our results are partially due to a very small sample size and the variability between individual crayfish.

Another possible explanation for our results could be that BAPTA-AM did not load into the motor neuron terminals. Our results showing that the initial EJP was smaller than that of the saline/DMSO control suggests that the BAPTA-AM loaded. However, the BAPTA and saline control initial EJPs were similar. Therefore, we cannot determine whether the BAPTA-AM permeated the cell and actively chelated intracellular calcium. However, Van der Kloot and Molgo (1993) used a method similar to ours for chelating intracellular calcium with BAPTA-AM and found it decreased synaptic facilitation in frog neuromuscular junctions.

However, even if the BAPTA did load, there is a further problem create4d by our method: BAPTA-AM can load into all cell types including presynaptic and postsynaptic cells. Some studies have found that intracellular calcium levels affect not only presynaptic but postsynaptic pathways as well. For instance, Wang and Kelly (1997) found that decreasing calcium by microinjection of BAPTA in the postsynaptic cell decreased depression. Hence, the nonspecificity of BAPTA-AM prevents us from relating BAPTA-AM's effects to residual calcium in only the presynaptic cell.

Our unanticipated results could also be related to the temporal relationship between facilitation and depression. Specifically, facilitation *decreases* as the delay between stimuli increases, whereas depression *increases* as the delay between stimuli increases (Zucker, 1999). The delay times that we chose, 25 and 35 msec, could be located near the intersection of the facilitation/depression relationship, thus explaining the opposite results obtained. In order to test this relationship, time delays with a greater temporal difference, such as 25 msec and 100 msec, should be studied.

In future experiments, a greater sample size should be used in order to alleviate statistical problems encountered with a small sample size. There is a large degree of variability between individual crayfish, so using large sample sizes may correct for the discrepancies in EJP amplitudes. Such discrepancies are exemplified in our saline and saline/DMSO controls; theoretically, these two treatments should yield the same results, yet they were significantly different. Furthermore, the microinjection of BAPTA would insure that the chelator affected only the presynaptic cell. This procedure would allow us to specify whether calcium levels within the presynaptic cell are responsible for facilitation. Further work using BAPTA-AM could involve the use of a fluorescent tag to insure that the chelator was loaded. Additionally, we may need to perform analyses using different calcium chelators in order to determine which works best under our experimental conditions. Both EGTA and BAPTA have been used extensively in examining the effects of chelators on plasticity, and many of these studies show conflicting results (Kamiya and Zucker, 1994). In addition, BAPTA is a fast chelator that may affect the initial EJP; therefore, further research about facilitation may yield more conclusive results if a slow chelator is used.

REFERENCES

Blundon, JA, SN Wright, MS Brodwick, GD Bittner. 1993. Residual free calcium is not responsible for facilitation of neurotransmitter release. *Proc. Natl. Acad. Sci.* USA. 90:9388-9392.

Cummings, DD, KS Wilcox, and MA Dichter. 1996. Calcium-dependent paired-pulse facilitation of miniature EPSC frequency accompanies depression of EPSCs at hippocampal synapses in culture. *J Neurosci.* 16(17):5312-5323.

Hsu, KS, WC Ho, CC Huang, and JJ Tsai. 1999. Prior short-term synaptic disinhibition facilitates long-term potentiation and suppresses long-term depression at CA1 hippocampal synapses. *Eur J Neurosci.* 11(11):4059-69.

Kamiya, H and RS Zucker. 1994. Residual Ca2+ and short-term synaptic plasticity. *Nature.* 371(13)603-606.

Lindgren, CA, DG Emery, PG Haydon (1997) Intracellular acidification reversibly reduces endocytosis at the neuromuscular junction. *J Neurosci* 17(9):3074-3084.

Ouanounou, A, L Zhang, MD Charlton, and PL Carlen (1999) Differential modulation of synaptic transmission by calcium chelators in young and aged hippocampal CA1 neurons: evidence for altered calcium homeostasis in aging. *J Neurosci* 19(3):906-915.

Parker, D (2000) Activity in calcium-dependent mechanisms maintain reliable interneuron synaptic transmission in a

rhythmic neural network. *J Neurosci* 20(5):1754-1766.

Stephens, Philip J (1996) Teaching Physiology with MacLab and Mac: the Crayfish superficial flexor muscle. HyperCard 2.1.

Tymianski, M, MP Charlton, PL Carlen, and CH Tator (1994) Properties of neuroprotective cell-permanent Ca2+ chelators: effects on [Ca2+]i and glutamate neurotoxicity in vitro. *J Neurophysiol* 72(4):1973-92.

Van der Kloot, W and J Molgo (1993) Facilitation and delayed release at about 0 degree C at the frog neuromuscular junction: effects of calcium chelators, calcium transport inhibitors, and okadaic acid. *J Neurophysiol* 69(3):717-29.

Wang, JH and PT Kelly (1997) Postsynaptic calcineurin activity downregulates synaptic transmission by weakening intracellular Ca2+ signaling mechanisms in hippocampal CA1 neurons. *J Neurosci* 17(12):4600-11.

Xie, X, G Barrionuevo, and TW Berger (1996) Differential expression of short-term potentiation by AMPA and NMDA receptors in dentate gyrus. *Learn Mem 3*(2-3):115-23.

Zucker, RS (1989) Short-term synaptic plasticity. *Annu Rev Neurosci* 12:13-31.

Zucker, RS, DM Kullmann, and M Bennett (1999) Release of Neurotransmitters. In: *Fundamental Neuroscience*. (Zigmond, Bloom, Landis, Roberts, and Squire, ed), pp 155-192. New York: Academic Press.