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Citrate and NTA not suitable as extracellular calcium buffers at the crayfish neuromuscular junction

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ABSTRACT

Previous research has suggested that the calcium buffers citrate and/or nitrilotriacetic acid (NTA) may alter neurotransmitter release at the neuromuscular junction, allowing researchers to investigate the role of fluctuations in calcium concentrations outside the pre-synaptic terminal. To examine the effects of these chemicals, this research set out to measure changes in excitatory post-synaptic potentials (EPSPs) caused by citrate and NTA at the crayfish neuromuscular junction. Using standard intracellular recording and extracellular stimulation techniques, we measured EPSP amplitudes in magnesium-free, low calcium Ringers solutions and in two solutions with citrate and NTA added separately. We experienced great difficulty in recording control data and were unsuccessful in recording any data for the buffered solutions. This result suggests that the lowered calcium concentration (required for NTA and citrate to act as buffers) combined with the lack of magnesium in the solutions interferes with the normal stimulation of an EPSP. This calls into question the suitability of citrate and NTA as calcium buffers at the crayfish neuromuscular junction.

INTRODUCTION

The influx of external calcium into the presynaptic terminal plays an integral role in the release of neurotransmitters from the presynaptic terminal (Dittman & Regehr, 1998). In certain cells, the influx of calcium has been shown to cause localized decreases in extracellular calcium concentration (Hilgemann, 1983). To better understand the effects of extracellular calcium concentration on synaptic signaling, Ginsburg and Rahamimoff (1983) used citrate to maintain a constant level of calcium outside the frog neuromuscular junction. Citrate acts as a calcium buffer at low levels of calcium (0.3 to 1.05 mM) (Ginsburg & Rahamimoff, 1983), maintaining a constant calcium concentration despite the addition or removal of calcium in the solution. Thus, citrate should prevent the normally observed decrease in extracellular calcium through its buffering action. This in turn should cause an increase in excitatory post-synaptic potential (EPSP) amplitude following a stimulus train because more calcium is available to enter the cell and facilitate neurotransmitter release. Unexpectedly, Ginsburg and Rahamimoff found a decrease in EPSP strength, indicating a reduction in extracellular calcium.

Later studies, however, suggested that citrate interferes with calcium channels in rabbit ventricular myocytes rather than acting solely as a calcium buffer (Bers et al., 1991; Bers et al., 1992). These studies used nitrilotriacetic acid (NTA) as an alternative calcium buffer and found that NTA caused significantly different results than citrate. Bers et al.'s (1991) results for NTA were in line with those expected for a calcium buffer. These results bring into question the use of citrate as a calcium buffer in biological systems. At the same time, they provide an alternative buffer (NTA) with which to test the role of extracellular calcium on neurotransmitter release.

Our research sought to find whether NTA and citrate act on the crayfish neuromuscular junction as predicted by Bers et al. (1991). Using intracellular recording, we planned to stimulate the nerve and measure the EPSPs in zero-magnesium, low-calcium Ringer's saline, followed by measurement in saline containing either citrate or NTA. A magnesium-free solution was necessary because magnesium can negativity interact with calcium buffers (Bers et al., 1991). After stimulating the nerve at a high frequency, we planned to again measure EPSPs in the saline solutions. This stimulus train was included in the procedure to observe the effects of decreased extracellular calcium. Although we hypothesized that our results would agree with Bers et al. (1991) in that citrate decreases the EPSPs while NTA does not cause a change in EPSPs, we were unable to either confirm or reject our hypothesis due to great difficulty in recording EPSPs in low calcium solutions. This result may indicate that low calcium concentrations reduce the likelihood of eliciting an EPSP, questioning the use of NTA and citrate as calcium buffers at the crayfish neuromuscular junction.

MATERIALS AND METHODS

Preparation

Our experiment used the neuromuscular junction at the superficial flexor muscle in the tail of *Procambarus clarkii* crayfish. The crayfish were placed in ice for at least 15 minutes before dissection to minimize suffering.

Solutions

Five different saline solutions were used in our experiment: normal Ringer's saline, low calcium and zero magnesium control solutions, and saline containing either sodium-citrate or nitrilotriacetic acid (NTA) (See Table 1). All solutions had a pH of 7.4 obtained by the addition of HCl. We changed solutions approximately every 20 minutes in order to maintain cellular viability of the crayfish tail.

Normal Ringer's Saline	Saline w/ Citrate	Control solution (before Citrate)	Saline w/ NTA	Control solution (before NTA)
205 NaCl	207.5 NaCl	227.5 NaCl	207.5 NaCl	227.5 NaCl
5.4 KCl	5.4 KCl	5.4 KCl	5.4 KCl	5.4 KCl
13.5 $CaCl_2$	1.0 $CaCl_2$	0.5 $CaCl_2$	1.0 $CaCl_2$	0.4 $CaCl_2$
10.0 TRIS	10.0 TRIS	10.0 TRIS	10.0 TRIS	10.0 TRIS
2.6 $MgCl_2$	10.0 Na-Citrate		10.0 NTA	

Table 1. Composition of solutions (all concentrations in mM). Final Ca^{2+} concentrations for buffered solutions found to be 0.5 and 0.4 for citrate and NTA, respectively, due to binding of calcium with buffer.

EPSPs were first obtained in normal Ringer's solution in two of our tests. As much of the saline as possible was then removed by pipette and replaced with control solution corresponding to the correct buffer. Once EPSPs were measured in this solution, the solution was again replaced, this time with the buffered solution, in which EPSPs were again elicited. The same nerve was used throughout.

We determined the appropriate levels of $[Ca]_o$ for the control solutions by creating curves using 50μM murexide and a spectrometer to find where each of the calcium buffers (citrate and NTA) lay on the curves (Figure 1). The saline solutions used to find the curves were magnesium-free and had levels of calcium ranging from 0 to 2mM. Two readings were done for each solution, at 470 nm and 542 nm (Scarpa, 1972).

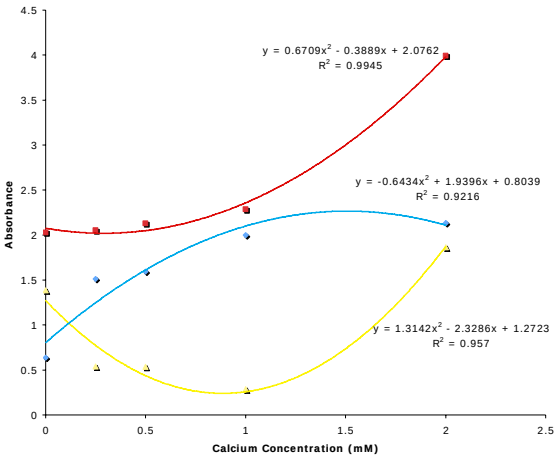

Figure 1. Calcium concentration as a function of absorbance for solutions containing murexide. Data points shown with quadratic fitted lines. Top line represents absorbance at 542 nm; middle line shows absorbance at 470 nm; bottom line shows difference between readings at 542 nm and 470 nm.

Cellular recording method

To measure the excitatory post-synaptic potentials (EPSPs) at the crayfish neuromuscular junction we used extracellular stimulating and intracellular recording equipment. A nerve was stimulated via a suction electrode, and a recording electrode inserted into the appropriate muscle cell measured the EPSPs. The recording electrode was connected to a voltmeter and filled with 3M KCl. If it had a resistance of less than 1.79 MΩ, the recording electrode was discarded and replaced.

EPSPs were first measured in the appropriate control solution, followed by the calcium buffer (either citrate or NTA). In two of the crayfish, we first placed them in normal Ringer's saline to find a neuromuscular junction that gave consistent EPSPs before putting the crayfish in the control solutions. Each of these two crayfish was placed in a different control solution.

In three tests of EPSPs in the citrate control solution, we stimulated the nerve at an average of 54.2 Hertz for ten seconds and recorded the subsequent EPSPs.

RESULTS

The original goal of this research was to investigate the role of extracellular calcium on neurotransmitter release as well as to determine whether citrate acted chemically in a manner other than as a calcium buffer. Despite many hours of work, we cannot specifically address these topics with the results we found. Our technique was based on stimulating and measuring excitatory post-synaptic potentials (EPSPs). However, we very rarely could elicit an EPSP in our experiment and we

were never able to when the tail was bathed in saline containing either citrate or nitrilotriacetic acid (NTA). Only three times did we obtain an EPSP in the control solutions, and two of those times were immediately after we found EPSPs in normal Ringer's saline. We had minimal trouble stimulating EPSPs when the crayfish tail was saturated in regular Ringer's saline.

We measured the amplitude of the EPSPs that we did see by comparing the resting membrane potential (rmp) to the highest point of the EPSP. The height of an EPSP in the 0.5mM Ca solution had a mean of 5.3mV, in 0.4mM Ca solution 5mV, and 1.9mV in normal Ringer's saline (standard error: 1.74, 0, 0.6, respectively).

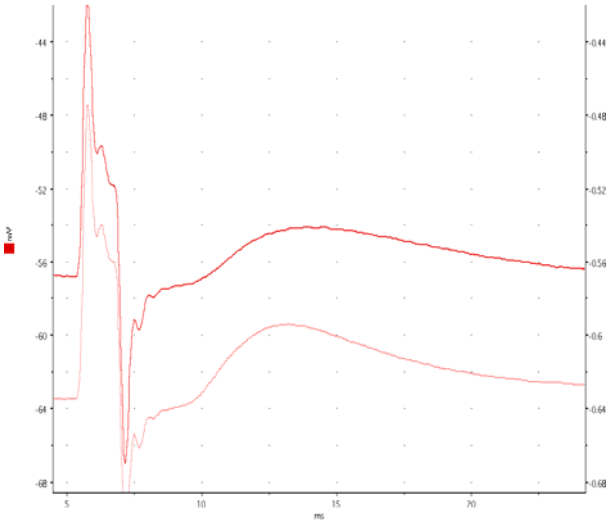

Figure 2. Bottom line shows the EPSP before the stimulus train at 54.2 Hz, while the top line shows the EPSP following in 0.5 mM calcium solution.

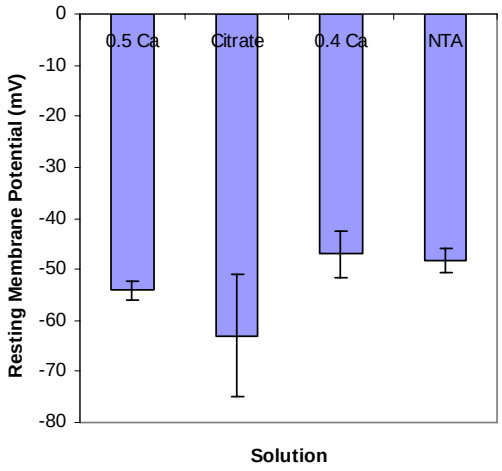

Figure 3. Bars show the resting membrane potentials in experimental solutions, and the standard error bars represent ± 1 S.E. For 0.5 Ca, n=23; for citrate, n=2; for 0.4 Ca, n=13; and for NTA, n=18. P > 0.5 in all cases.

Following a stimulus train in the three 0.5 mM Ca controls, the EPSPs changed. But without comparing this information to a stimulus train at 54.2 Hz in a buffered solution, no further data analysis can be performed. In two of the tests the EPSPs decreased by an average of 4.5mV (standard error 2), while the third test showed an increase of 1.5mV. Figure 2 shows the difference in EPSPs before and after a stimulus train.

No statistically significant differences between rmps in the various solutions was observed.

DISCUSSION

This experiment began on the premise that citrate would cause a decrease in excitatory post-synaptic potentials' (EPSP) magnitude while the addition of NTA would result in no change or a slight increase. Instead, our results show that EPSPs cannot be elicited in buffered solutions containing either citrate or NTA

Although our results cannot directly address our original questions due to the lack of EPSPs while using the citrate saline and the nitrilotriacetic acid (NTA) saline, our data does lead to interesting conclusions. Since citrate and NTA require a calcium concentration over ten times lower than that in normal Ringer's solution to be effective calcium buffers (Ginsburg & Rahamimoff, 1983), we found it very difficult to stimulate EPSPs at the crayfish neuromuscular junction. We spent many hours testing different nerves and penetrating different muscle cells along the crayfish tail to no avail, possibly because the calcium level was simply too low to elicit an EPSP. We were able to get three EPSPs in our low calcium control solutions, and two of those EPSPs occurred immediately after switching from the normal Ringer's saline, which has much higher calcium and magnesium concentrations. We suspect we were able to get an EPSP primarily because there was calcium and/or magnesium left over from the normal saline.

A study on crayfish neuromuscular junctions and the role calcium plays on EPSPs by Kawagoe et al. (1981) supports our conclusion that a certain amount of calcium is needed to elicit an EPSP. In the Kawagoe study, EPSPs were not recorded in many occasions when the magnesium was removed and Ca^{2+} concentrations were decreased to 5-10% of the normal solution. The calcium concentration of normal crayfish Ringer's solution is 13.5 mM. Citrate and NTA both require calcium concentrations over ten times less than that to function (Ginsburg & Rahamimoff, 1983) as well as no magnesium (Bers et al., 1991). For future experiments, we would use calcium buffers that could function in a much higher calcium concentration, instead of citrate and NTA.

Along with making it difficult to measure EPSPs, the extremely low calcium levels likely affected other aspects of the study. Ringer's solution is created to simulate the solution found inside the crayfish itself; lowering the calcium concentration to such a degree is straying from what is actually in the crayfish and therefore probably causes other changes within the cell. For instance, decreased $[Ca]_0$ reduces glutamate release at the crayfish neuromuscular junction (Kawagoe, 1981). Therefore it seems that if this subject is to be addressed further, studies must be performed at higher calcium concentrations.

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