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The Effect of Lanthanum as a Calcium Inhibitor on the CaSR-NSCC Pathway in the Crayfish Neuromuscular Junction

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ABSTRACT

Ca^{2+} is a key second messenger in many organisms; this include being essential for neurotransmitter release. Control of extracellular Ca^{2+} ($[Ca^{2+}]_o$) is critical in the human body; imbalances are associated with a large variety of neurological disorders. The Calcium Sensing Receptor (CaSR), the primary mediator in the Ca^{2+} sensor-nonselective cation channel signaling pathway, detects changes in $[Ca^{2+}]_o$, activating a Non-Selective Cation Channel (NSCC) to compensate neurotransmitter release as a response to changes in $[Ca^{2+}]_o$. Lanthanides have been found to inhibit NSCC in *Arabidopsis thaliana*. This study examines the effects of lanthanum on the NSCC in the crayfish neuromuscular junction. We found that the addition of lanthanum increased EPSP amplitudes and durations, thus failing to inhibit the NSCC. This contradicts the results seen in the *Arabidopsis thaliana* model, which may mean that lanthanides are unable to act as universal NSCC inhibitors or that the CaSR-NSCC signaling pathway operates differently in plants and invertebrate animals.

INTRODUCTION

Ca^{2+} works as a key messenger in a variety of organisms, acting intracellularly as a second messenger and extracellularly as a first messenger that is associated with neurotransmitter release (Brown, 2000). Extracellular Ca^{2+} ($[Ca^{2+}]_o$) represents less than one percent of all Ca^{2+} found in the human body, yet the control of this form of Ca^{2+} concentration is critical, with imbalances in $[Ca^{2+}]_o$ being associated with a large variety of neurological disorders (Harrington et al., 2007). Typically, in mammals, this extracellular ionized calcium is maintained at a physiological range of 1.1mM to 1.3mM, which is the level necessary for normal biological function (Harrington et al., 2007). Due to the importance of $[Ca^{2+}]_o$ and its regulation, a well-functioning regulatory system for monitoring $[Ca^{2+}]_o$ concentrations is necessary.

The calcium sensing receptor (CaSR) is the primary mediator of the mechanism controlling systemic $[Ca^{2+}]_o$ homeostasis (Brown, 2000). Recent studies have identified the CaSR as the first component in the CaSR-NSCC signaling pathway in neocortical cells synapses. It activates signaling pathways to maintain $[Ca^{2+}]_o$ homeostasis, such as parathyroid hormone secretion and parathyroid hyperplasia (Theman et al., 2009). Moreover, it has been shown that decreases in $[Ca^{2+}]_o$ are detected by the CaSR, which then increases action potential duration by increasing NSCC activity, thereby attenuating the impact of $[Ca^{2+}]_o$ decreases (Chen et al., 2010).

Inhibitors that work to regulate either the CaSR or NSCC in the CaSR-NSCC signaling pathway would allow for greater control of Ca^{2+} homeostasis. Demidchik et al. (2002) showed that common Ca^{2+} and K^+ channel antagonists, for example TEA, couldn't block the Ca^{2+} permeable NSCC. Other compounds and ions, such as Gd^{3+}, La^{3+}, spermidine, Ca^{2+} and Mg^{2+} can indirectly inhibit NSCC currents (Smith et al, 2004). Demidchik et al. (2002) previously showed that lanthanum inhibits NSCC currents in the plant species *Arabidopsis thaliana* (mouseear cress). The precise mechanism by which lanthanum inhibits NSCC remains unclear, but there is evidence to suggest that it positively modulates the GABA system by binding to a site near the surface of the GABA receptor-channel (Narahashi et al., 1994). The effects of inhibitors such as lanthanum, on the CaSR-NSCC signaling pathway has only been studied in plant species, therefore its effects on invertebrates and other model organisms is still unknown.

This study focused on testing whether the presence of lanthanum at different $[Ca^{2+}]_o$ in the neuromuscular junction of the *Procambarus clarkia* (crayfish) model would suggest similar results to those found in *Arabidopsis thaliana*, with lanthanum inhibiting NSCCs activity and $[Ca^{2+}]_o$ affecting the CaSR-NSCC signaling pathway. We found that the addition of lanthanum contradicted the results seen in the *Arabidopsis thaliana*, showing an overall trend of increasing EPSP amplitudes and durations. This is inconsistent with the blocking of NSCCs and the reduction of $[Ca^{2+}]_o$, which may mean that either lanthanides are unable to act as universal NSCC inhibitors or that the CaSR-NSCC signaling

pathway operates differently in plants and invertebrate animals.

MATERIALS AND METHODS

Dissection

Crayfish tails were utilized for our study in order to examine the effects of lanthanum on the action potentials produced by the crayfish tail extensor muscles. Crayfish tails were dissected with any unnecessary muscle being removed, exposing the lateral and medial tail extensor muscles along with small sections of motor nerves.

Solutions

The tails were placed in one of three different solutions of varying Ca^{2+} levels: 3.375 mM, 6.75 mM, and 13.5 mM (the standard Ca^{2+} testing level). These three solutions of differing Ca^{2+} concentrations acted as controls, allowing us to then compare these results to those we obtained using lanthanum in the bathing solution. Standard Ringer's solution (196mM NaCl, 5.4mM KCl, 13.5mM $CaCl_2$, 2.6mM $MgCl_2$, and 10mM Sodium Hepes Buffer maintained at pH 7.4) was used to prepare the different solutions. In diluted Ca^{2+} concentrations (3.375mM and 6.75mM), the $CaCl_2$ in the standard Ringer's solution was replaced with $MgCl_2$ of equal amounts. Additionally, a Ringer's solution with 0mM Ca^{2+} concentration (all $CaCl_2$ replaced with $MgCl_2$) was used as a test control. 1 mM Lanthanum nitrate solutions were produced using each of the three previously made Ca^{2+} solutions of varying concentrations: 3.375mM, 6.75mM, and 13.5mM (Demidchik, 2002). The lanthanum nitrate was purchased from Sigma Aldrich.

Microelectrodes

Using a PUL-1 electrode puller, we made microelectrodes in order to record excitatory post-synaptic potentials (EPSP) in postsynaptic cells. The microelectrodes were created using 1.2mm diameter glass capillary tubes. The microelectrodes were then filled with 3M KCl in order to conduct the recordings. The resistance of these microelectrodes ranged from approximately 4 to 10MΩ.

Recording

EPSPs were obtained for tails in each solution, using suction electrodes to electrically stimulate a presynaptic nerve using a GRASS SD9 stimulator. The stimulator fired single repeating pulses at a frequency of 0.5 Hz in order to produce EPSPs. EPSPs were measured at the lowest possible voltage using glass microelectrodes. For each concentration of Ca^{2+}, we recorded three readings at different locations on the extensor muscle. After obtaining EPSPs for the three Ca^{2+} solutions, we examined the changes in EPSP using lanthanum with our three different Ca^{2+} solutions (3.375mM, 6.75mM, and 13.5mM). The identical recording process was repeated for each solution. Data was analyzed using the program SCOPE in order to measure the height and length of the EPSPs.

RESULTS

The purpose of this research was to study the effect of lanthanum on the NSCC in the crayfish neuromuscular junction. To study this, we measured the amplitude and duration of EPSPs at three different calcium concentrations. Our data suggest that the effect of lanthanum on the NSCC differs from those seen by Demidchik et al. (2002) in *Arabidopsis thaliana*.

The data indicate that both duration and amplitude of EPSPs increased with addition of 1mM lanthanum to the bathing solution (Fig 1). We measured the EPSPs in the solutions with varying $[Ca^{2+}]_o$ and then repeated the measurements with addition of lanthanum. At 3.375mM and 13.5mM $[Ca^{2+}]_o$ we observed an increase in the duration and amplitude of EPSPs, calculated as a percentage increase from the solutions with no lanthanum present (Fig 2). The average EPSP amplitude at 3.375mM $[Ca^{2+}]_o$ increased from 1.56 to 6.04mV with addition of lanthanum. Similarly, the average EPSP at 13.5mM increased from 6.11 to 12.35mV after lanthanum was added to the solution (Fig 2).

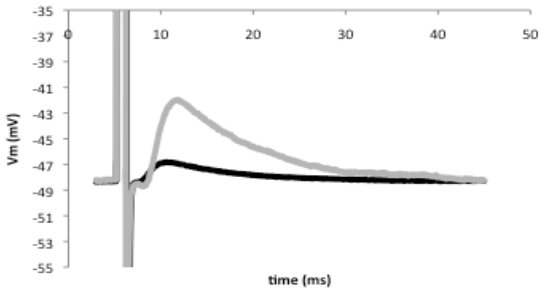

Figure 1. EPSPs with lanthanum show longer duration and greater amplitude. Representative recording of EPSPs. This EPSP is for 3.375mM $[Ca^{2+}]$ with and without lanthanum. The larger and longer lasting EPSP recording shows the results of one trial with lanthanum (gray), while the other EPSP is without lanthanum (black). These trials were repeated for each $[Ca^{2+}]_o$ (n=3). The EPSP for $[Ca^{2+}]_o$ without lanthanum has been increased by 2mV for display purposes

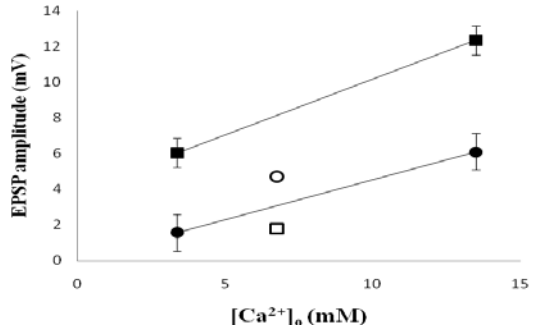

Figure 2. Lanthanum increases EPSP amplitude with increasing $[Ca^{2+}]_o$. Average traces of EPSP amplitudes at 3.375 mM and 13.5 mM $[Ca^{2+}]_o$ only (●) are lower compared to those with 1mM Lanthanum (■). The linear fit does not include data points at 6.75mM $[Ca^{2+}]_o$ (○ &□) because data points obtained had lower voltage and were inconsistent. Results show the average amplitude increase for each concentration (n=3). Standard error bars = SEM.

Moreover, we noted that the solution with lanthanum showed a larger percentage increase in the lower $[Ca^{2+}]_o$ than the 13.5mM concentration for amplitude (Fig 3). There was no significant difference between the duration for the two concentrations, although there was still an overall increase in duration with lanthanum (Fig 4). However, in the 6.75mM $[Ca^{2+}]_o$ with lanthanum we observed a decrease in the amplitude and duration of EPSPs as compared to the 6.75mM $[Ca^{2+}]_o$ without lanthanum, which is not consistent with our other results (Fig 2). Additionally, more substantial variances were seen between the two tests at this concentration than were seen among the tests for other concentrations. Due to inconsistent and conflicting recordings as well as the data obtained from our 6.75mM $[Ca^{2+}]_o$ solution, we have chosen to focus on our more constant results seen with the 3.375mM and 13.5mM $[Ca^{2+}]_o$ solutions.

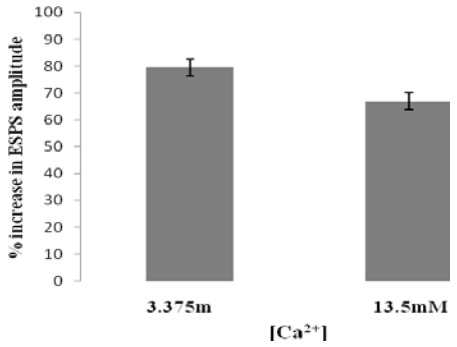

Figure 3. EPSP amplitude increases with lower $[Ca^{2+}]_o$ in lanthanum solutions. The percentage increase in EPSP amplitude in the presence of 1mM Lanthanum at 3.375mM and 13.5mM $[Ca^{2+}]_o$. Results show the average amplitude increase for each concentration (n=3). Standard error bars = SEM.

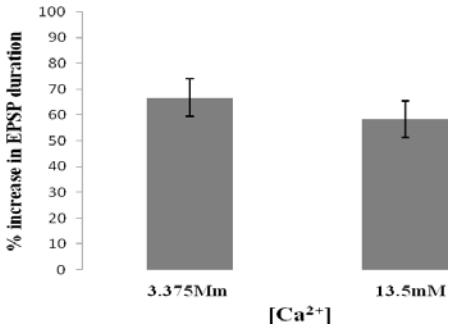

Figure 4. EPSP duration increases with lower $[Ca^{2+}]_o$ in lanthanum solutions.. The percentage increase in EPSP duration with the addition of 1mM Lanthanum at 3.375mM and 13.5mM $[Ca^{2+}]$. Results show the average amplitude increase for each concentration (n=3). No significant difference was seen between $[Ca^{2+}]_o$. Standard error bars=SEM.

DISCUSSION

Our results indicate that lanthanum does not inhibit NSCCs as expected and as previously seen in the study by Demidchik et al. (2002) on *Arabidopsis thaliana*. This may indicate that either the CaSR-NSCC signaling pathway functions differently in plants than in invertebrate animals or that lanthanides do not produce the same effects on the NSCC in invertebrates as those previously seen in plants. We studied the amplitude and duration of EPSPs at different $[Ca^{2+}]_o$ with 1mM lanthanum and found the EPSPs to increase in both duration and amplitude.

In our EPSP recordings, amplitude increased with lanthanum at both concentrations (Fig 3), as did EPSP duration when lanthanum was added (Fig 4). The lower $[Ca^{2+}]_o$ shows a larger percentage increase for amplitude when compared to the higher $[Ca^{2+}]_o$, which is consistent with results found by Chen et al. (2010) for the use of an agonist on the CaSR in mice. Chen et al. (2010) suggests that increased NSCC activity may increase action potential duration because it prolongs Ca^{2+} entry at the terminal. This contradicts our results in which lanthanum caused an increase in EPSP duration. This divergence implies that lanthanum did not inhibit NSCC activity, which would have reduced EPSP duration.

Despite consistent results seen at 3.375mM and 13.5mM Ca^{2+} concentrations, we observed an anomaly at the 6.75mM $[Ca^{2+}]$, where the addition of lanthanum resulted in a decrease of amplitude and duration. This may be due to our only being able to obtain two data points for this concentration, which might misrepresent the average. Thus, we did not include the 6.75mM data points in the majority of our data analysis and focused primarily on the two remaining concentrations with more constant results. Additionally, Demidchik, Davenport et al. (2002) found that the low selectivity of NSCCs pose a

challenge for electrophysiological studies because of the difficulty associated with identifying the ionic species that is responsible for the current being produced. This may further contribute to our lack of consistent data seen in some of our recordings.

The differences seen in the effects of lanthanum on the crayfish neuromuscular junction as compared to those seen in *Arabidopsis thaliana* may reflect either variations in the CaSR-NSCC pathway or differences in the way in which lanthanides specifically affect the pathway. Both of these theories may be further explored by the utilization of other inhibitors or agonists for either the NSCC or CaSR in the crayfish neuromuscular junction. Our study had initially also intended to explore the effects of Calindol, a CaSR agonist, but was unable to due to a lack of time. Use of Calindol or other inhibitors or agonists of the pathway could allow for further understanding of the CaSR-NSCC signaling pathway in an invertebrate model. Additionally, examining the effects of lanthanum in vertebrates would allow for a better understanding of lanthanum's overall effects and the mechanism by which it is able to act as an inhibitor.

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