PHARMACOLOGICAL IDENTIFICATION OF A CALCIUM RELEASE CHANNEL IN THE ENDOPLASMIC RETICULUM OF PLANTS

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

ABSTRACT

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Whenever we contract our muscles, we do so because there is an ion channel in the endoplasmic reticulum (ER) that releases calcium from within the ER in response to an action potential. Plants do not have the genes for the animal calcium release channels, but they, nevertheless, release calcium from their ER. When *Arabidopsis thaliana* is subjected to photostimulation in the ER-chloroplast nexus, a calcium wave is observed in the cytoplasm of the cell. Our hypothesis is that an ER calcium release channel is responsible for the calcium wave. We hypothesize that gadolinium blocks at least one of two suspected calcium release channels involved in the production of the calcium wave following photostimulation. We test these hypotheses by examining which part, if any, of the calcium wave is inhibited by gadolinium.

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NOMENCLATURE

ER	Endoplasmic Reticulum
YFP	Yellow Fluorescent Protein
CFP	Cyan Fluorescent Protein

CHAPTER I

INTRODUCTION

The Calcium Wave

Plants are organisms that are incapable of locomotion. Despite the fact that they are incapable of changing their surroundings, they are occasionally forced to react to stressors including pathogenic attack, disruptions in soil ion balance, and extreme changes in temperature inducing cold or heat shock. Recently, a calcium wave has been observed in response to various biological stressors. The calcium wave is thought to be a root-to-shoot signaling event, responsible for informing the rest of the plant of the stress by increasing the stress signals and transcription of stress genes (Choi, 2014). In work that has yet to be published, Dr. Griffing's lab has found that focusing high-intensity 405 nm blue light on the ER-chloroplast junction of individual *Arabidopsis thaliana* cells is sufficient for induction of an intracellular calcium wave.

The endoplasmic reticulum is an organelle in eukaryotic cells that plays a key role in the release of the second messenger, calcium. We believe that there is a voltage-gated ion channel in the endoplasmic reticulum that is responsible for the release of calcium, and that this ion channel can be inhibited using pharmacological inhibitors. By inhibiting the voltage-gated ion channel, it will be possible to prevent the progression of the calcium wave.

While it is believed that the calcium wave is a result of calcium released from the endoplasmic reticulum, there may be other sources of calcium involved in the calcium wave. It has been shown that eosin Y is capable of inhibiting calcium efflux across the plasma membrane of plants. It has also been shown that external ATP in combination with L-glutamate is capable of causing a substantial increase in the concentrations of cytosolic calcium (Bonza et al., 2013).

The calcium wave may consist of a combination of calcium from the endoplasmic reticulum, and contributions from plasma membrane influx.

Pharmacological Inhibitors

The calcium wave has been shown to be affected by various pharmacological inhibitors. A known inhibitor of calcium ATPases in the sarcoplasmic reticulum of cells is cyclopiazonic acid. By inhibiting calcium ATPases, cyclopiazonic acid is capable of blocking calcium reuptake in cells (Johnson, 2009). Similarly, it is thought that gadolinium is a pharmacological agent that can be used in an analogous manner to block the calcium release from the endoplasmic reticulum of *Arabidopsis.* In *Bryonia diocia*, a plant different from the one that we use, gadolinium has been used to inhibit the ER voltage-dependent calcium release channels that are responsible for the calcium-driven response of leaf tendril curling. Electrophysiological measurements confirmed that the gadolinium is what prevented the calcium from entering the voltage-dependent calcium release channels (Klüsener, 1995). Liang demonstrated that gadolinium is a successful inhibitor of endoplasmic reticulum voltage-dependent calcium channels in yeast expressing the *Arabidopsis ECA1* gene (Liang, 1998). If we are able to show that gadolinium is a specific inhibitor of the voltage-gated ion channels responsible for the release of calcium into the cytosol of *Arabidopsis* cells in response to photostimulation, then we will be able to determine whether or not a release channel is present, and whether or not it responsible for producing the calcium wave.

CHAPTER II

METHODS

Plants

All of the experiments were performed using five-day-old *Arabidopsis thaliana* seedlings that were grown under 24 hour light. These seedlings transgenically express YCNano-65, a cytosolic calcium reporter gene. YCNano-65 has calcium-binding domains that induce conformational changes, which allow for the measurement of calcium.

Pharmacological Inhibitors

Seedlings were treated with 0.1mM, 1mM, and 10mM gadolinium solutions, and a control seedling was treated with 10 mM MES buffer alone. The gadolinium solutions contained gadolinium and 10mM MES buffer. The 10mM MES buffer was prepared by adding 233mg of potassium salt to 10mL of double distilled water. This produced 100mM MES buffer. HCl was added until the pH of the buffer was 5.8. A 1:10 dilution was then performed to get the final concentration of 10mM of MES buffer. Some MES buffer was set aside for the incubation of the control seedling. To make the gadolinium solutions, enough gadolinium was added to 10mM MES buffer to make a 10mM gadolinium and MES buffer solution. To prepare the 1mM gadolinium solution, a 1:10 dilution was performed on the 10mM gadolinium solution, using 10mM MES buffer to get a final concentration of 1mM. Finally, to prepare the 0.1mM gadolinium solution, a 1:10 dilution was performed on the 1mM gadolinium solution using 10mM MES buffer to get a final concentration of 0.1mM.

There were two seedlings for each solution. The seedlings were allowed to incubate in their respective solution for 30 minutes before photostimulation. The seedling incubated in MES buffer only was used as a control to observe a typical calcium wave. The level of inhibition produced by the different concentrations of gadolinium was determined by comparing those calcium waves to the calcium wave of the MES buffer only seedling.

Photostimulation

The seedlings were photostimulated using the Olympus FV1000 confocal microscope in the Microscopy and Imaging center at Texas A&M University. First, plants were screened in MES buffer to check for expression of YCNano-65. Plants with high expression of YCNano-65 were chosen for the experiments. Each seedling that was chosen was then assigned to a 0.1mM gadolinium, 1mM gadolinium, or 10mM gadolinium, or MES buffer only solution. The photostimulation was performed after each seedling had incubated in its respective gadolinium or MES buffer solution for at least 30 minutes. After a 30-frame delay, a 405nm SIM laser with 100% power was used to photostimulate hypocotyl cells over a 10x10 pixel region of interest in the area where the ER meets the chloroplast, also known as the ER-chloroplast nexus. The response was recorded over 400 frames, at 0.4 seconds per frame.

Image Analysis

The images obtained from the confocal microscope were processed using FIJI to obtain quantitative data that could later be used to make graphs in Microsoft Excel. The confocal microscope produced a video of the 400 frames recorded. A ratio of YFP fluorescence to CFP fluorescence was obtained by dividing the images obtained from the YFP channel into the

images obtained from the CFP channel. A one-pixel radius median filter was applied to reduce noise. A lookup table called Green Fire Blue was applied to the resulting image to assist in visualization of the calcium wave. This lookup table makes high calcium concentrations appear green, and low calcium concentrations appear blue. The image was rotated so that the cell of interest was vertical, and then a 5.8-micron (14 pixel) wide section of the calcium wave was cropped. The cropped section was centered around the photostimulated chloroplast. From the resulting cropped section, a montage of the 400 frames was made, and the resulting montage was then graphed, resulting in a kymograph. The kymograph consisted of the YFP to CFP ratio (grey value) plotted against the distance of the montage. From the kymograph, a list of values was obtained showing the grey value for every pixel (or 0.41 micrometer). The grey values were then processed in Microsoft Excel. Because there are 56 pixels in four 5.8 micrometer-wide slices, the grey values were averaged over 56 pixels. Each frame represented 0.4 seconds; so four slices averaged represented 1.6 seconds.

After several repetitions of the experiments, the final grey values obtained after all of the image processing were averaged together. The averages of the grey values were then plotted to obtain a graph of ratio value plotted against time. Control images were processed in the same way, and these were added to the graph so the calcium wave produced after exposure to gadolinium could be compared to them.

CHAPTER III

RESULTS

Measurement of the Calcium Wave

The seedlings were incubated in gadolinium and photostimulated in the way described in the materials and methods section. Once the cells were photostimulated at the ER-chloroplast nexus, a calcium wave that spanned the entire cell was seen. Figure 1 shows a cell before and after photostimulation. Quantification and therefore better utilization of the calcium wave was possible from using image processing. The cell after photostimulation was used for image processing (as shown on right in Figure 1). Further processing, as described in the materials and methods section, resulted the montage (Figure 2. A), kymograph (Figure 2. B), and Microsoft Excel graph (Figure 2. C), shown below.

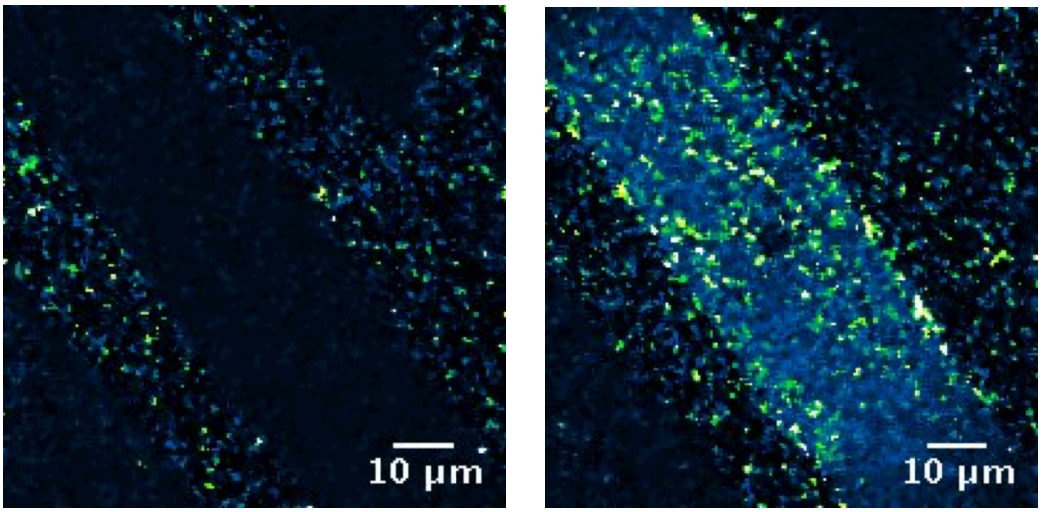
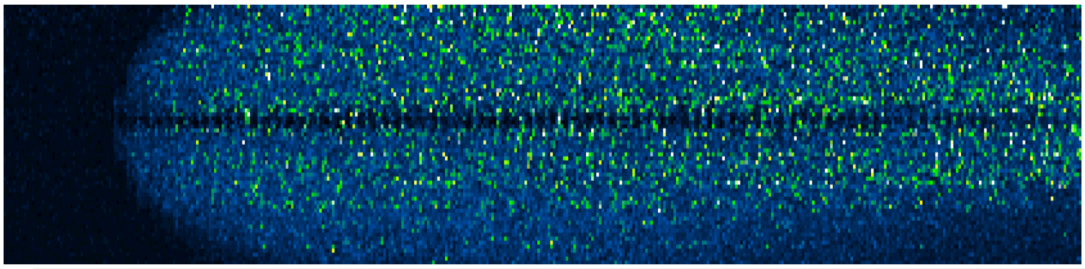


Figure 1. A hypocotyl surface cell of a 5-7 day old *Arabidopsis thaliana* seedling. The seedling was incubated approximately 55 minutes in 0.1mM gadolinium before the images were obtained. The image on the left shows the seedling before photostimulation. The image on the right shows the same seedling after photostimulation in the ER-chloroplast nexus. The photostimulated chloroplast is the dark spot in the middle of the cell.

A



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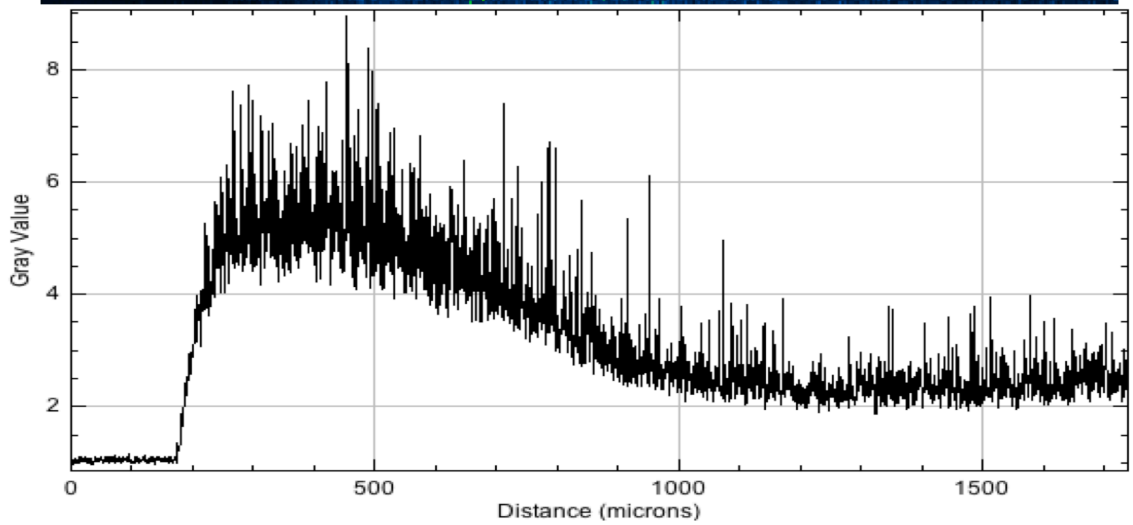

C

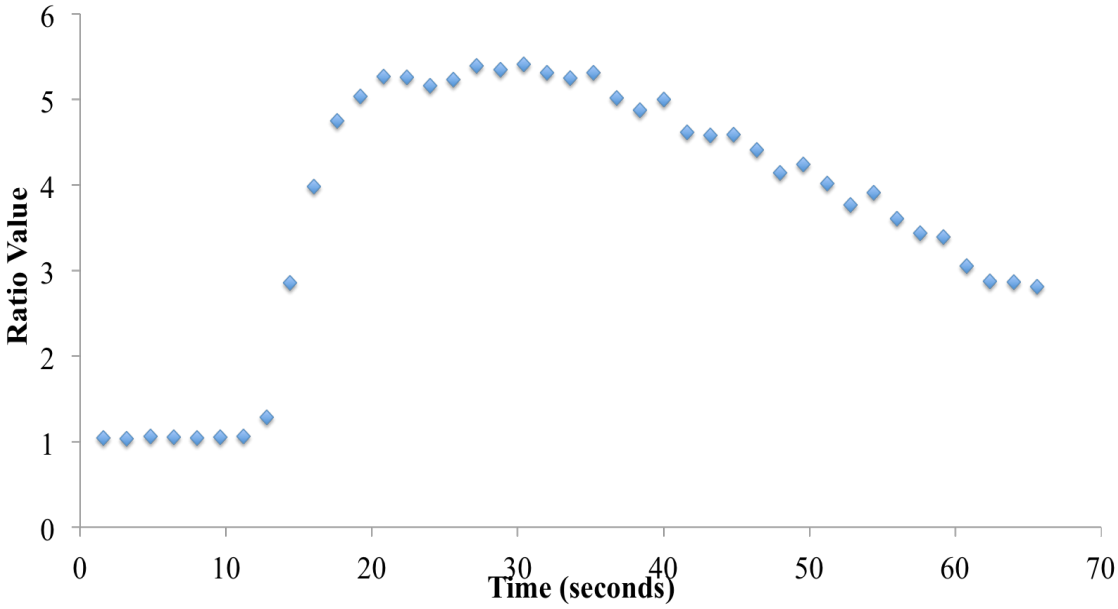

Figure 2. A) The video from the confocal microscope of the calcium wave produced by the seedling in 0.1mM gadolinium was processed in ImageJ. It was cropped into a 5.8 micron section (14 pixels) and was centered around the chloroplast. The dark line down the middle of the montage is the chloroplast. The cropped area was made into a montage using FIJI. B) The montage from part A was plotted into this kymograph. The kymograph plots the ratio of YFP fluorescence to CFP fluorescence (grey value) against the distance of the montage (in microns). C) The grey values from the kymograph were averaged over 56 pixels and plotted against time to obtain the ratio value. The ratio value was then plotted against the time in seconds.

After image processing, we were able to observe an increase in cytosolic calcium following photostimulation before calcium levels plateaued and eventually dropped off (Figure 2C). These figures show the calcium wave produced by a seedling in the lowest concentration of gadolinium (0.1mM); if gadolinium is an inhibitor of calcium release channels, there may have been some inhibitory effect. Results similar to these were obtained for all of the photostimulated seedlings, including the control.

Effects of Gadolinium on the Calcium Wave

Three different concentrations of gadolinium were used. Seedlings were initially screened in MES buffer for expression of CFP and YFP. Seedlings with high levels of expression were then incubated in their respective concentrations of gadolinium for about 55 minutes before photostimulation. Using the image processing techniques previously described in the materials and methods section, it was possible to quantify the cytosolic calcium waves produced by each seedling. The ratios of the cytosolic calcium increases were then plotted to see if the presence of gadolinium had an effect on the resulting calcium wave that was produced. The figure below (Figure 3) shows the averages obtained from several repetitions of these experiments. Due to variations in the natural baseline cytosolic calcium levels of plants, the initial ratio values of some plants concentrations of gadolinium differ. The grey value of the 10mM concentration of gadolinium has an initial ratio value of 2 because the seedlings treated with 10mM gadolinium had higher baseline calcium values compared to the seedlings treated with the 0.1mM and 1mM concentrations of gadolinium. Standard error bars are omitted from the graph in Figure 3 because the experiments are still in the process of being reported in order to obtain better standard deviations.

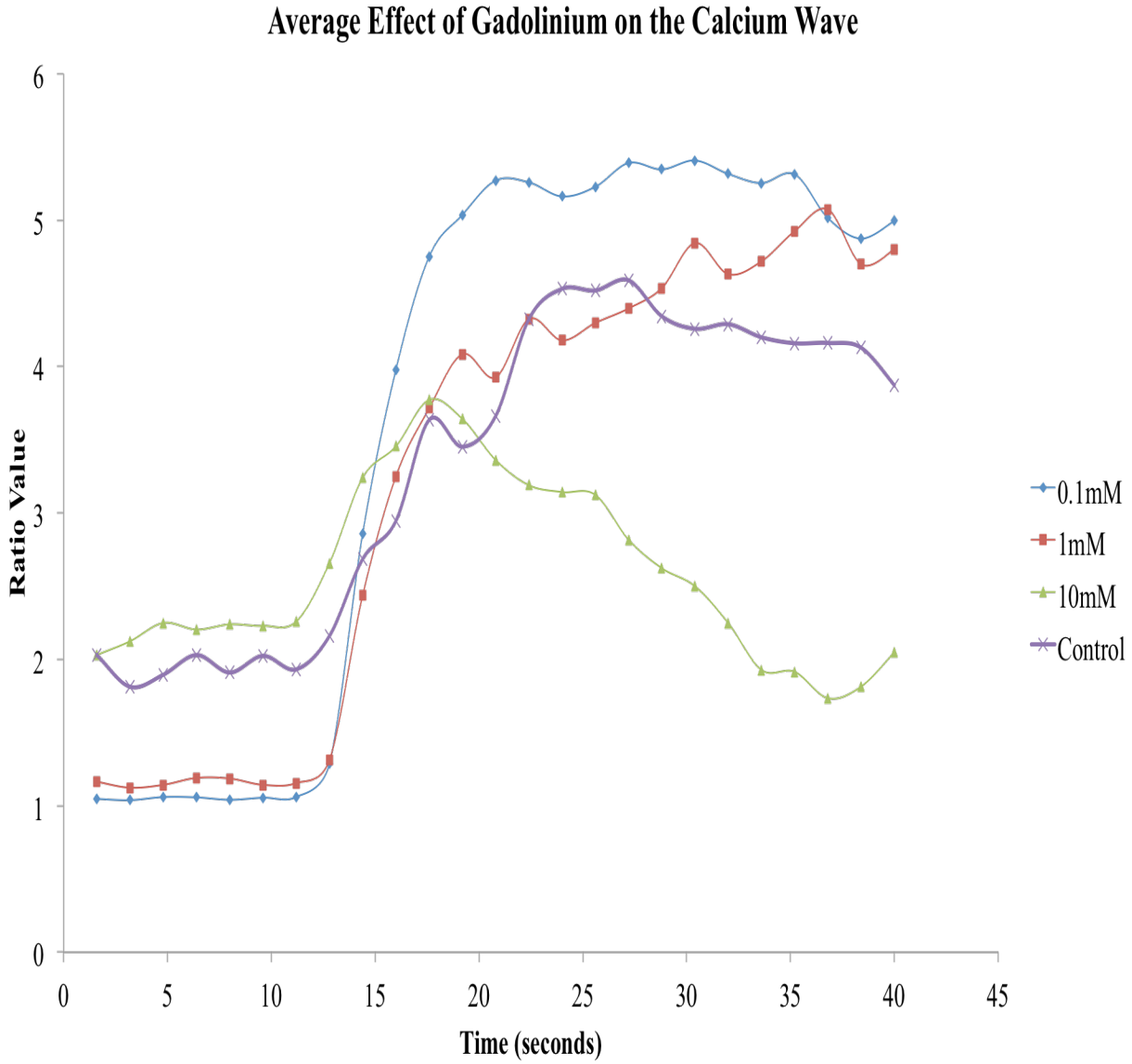

Figure 3. Seedlings were incubated in 0.1mM, 1mM, and 10mM concentrations of gadolinium for about 55 minutes before photostimulation. The control was incubated in MES buffer only for 55 minutes before photostimulation. The average ratio values of cytosolic calcium levels from several experiments were averaged together to produce this graph. This graph shows the average ratio value of cytosolic calcium for a given point in time.

Table 1: Comparison of the ratio values across the various concentrations of gadolinium.

Ratio Value at Time (Seconds)	Control	0.1mM	1mM	10mM
1.6	2.028	1.047	1.163	2.030
20.8	3.661	5.270	3.926	3.362
40	3.872	4.995	4.800	2.049
Cytosolic Calcium Increase	1.633	4.223	2.763	1.332

Note: The increase in cytosolic calcium was calculated by subtracting the ratio value at 1.6 seconds from the ratio value at 20.8 seconds. The blue highlighting indicates that this was the smallest cytosolic calcium increase, and the green highlighting indicates the highest cytosolic calcium increase.

The different concentrations of gadolinium caused varying levels of inhibition of the calcium wave, as was shown by varying levels of cytosolic calcium following photostimulation. The lowest concentration of gadolinium, 0.1mM, showed no inhibitory effect. The much larger increase in calcium in comparison to the control values suggests that 0.1mM of gadolinium may have even had a slightly stimulatory effect (Figure 3). The 1mM concentration of gadolinium did not show much of an effect as compared to the control values. The calcium increases are very similar in the two. The 10mM concentration of gadolinium shows an increase in cytosolic calcium, but less so than the control and all of the other concentrations of gadolinium. Table 1 takes the data in Figure 3 and looks at the ratio values at certain points of time. From this, we were able to see that the 10mM concentration of gadolinium caused a smaller increase in calcium levels than the control, while the other 0.1mM and 1mM concentrations of gadolinium did not have an inhibitory effect.

Summary

Plants were incubated in their respective concentrations of gadolinium for approximately 55 minutes before photostimulation. A control value was obtained by incubating a plant in MES

buffer for the same amount of time before photostimulation. The images obtained from the confocal microscope were processed and quantified. The values obtained from the quantification were averaged together to produce a graph of the grey values plotted over time. The values of each concentration of gadolinium were compared to the control value to determine if a particular concentration of gadolinium had a stimulatory or inhibitory effect.

The table below (Table 2) gives a summary of the overall effects of gadolinium on the channel. The 0.1mM and 1mM concentrations of gadolinium showed an increase in the cytosolic calcium ratios similar to the control, and did not show an inhibitory effect. The 10mM concentration of gadolinium did show an inhibitory effect, because although there was an increase in the concentration of cytosolic calcium, the increase in cytosolic calcium was smaller than the increase in calcium seen in the control values.

Table 2: The proposed overall effect of the different concentrations of gadolinium on the calcium wave.

Concentration of Gadolinium	ER Channel	Effect on Calcium Wave (as compared to the control values)
0.1mM	Not Inhibited	None
1mM	Not Inhibited	None
10mM	Inhibited	Inhibitory

Note: In order to determine if a concentration of gadolinium had an inhibitory effect or not, the calcium drops for each concentration (table 1) were compared to the calcium drop seen for the control values. The only concentration of gadolinium found to have an inhibitory effect compared to the control values was the 10mM concentration of gadolinium.

CHAPTER IV

CONCLUSION

Effectiveness of Gadolinium as an Inhibitor

Gadolinium was shown to be an effective inhibitor of voltage the calcium wave. 0.1mM and 1mM concentrations of gadolinium were expected to be relatively ineffective at preventing an increase in cytosolic calcium. As predicted, the lower concentrations of gadolinium appeared to have little to no effect on the calcium wave, but the 10mM concentration showed a reduction in cytosolic calcium levels after photostimulation.

Although the 10mM concentration of gadolinium was shown to be effective in inhibiting the calcium wave, there was still a small increase in the cytosolic calcium levels after photostimulation. However, this may not be evidence that our conclusion is wrong and that gadolinium is actually an ineffective inhibitor. Instead, this may be evidence that the calcium in the calcium wave is drawn from sources other than just the endoplasmic reticulum. There are proton-calcium antiporters and calcium ATPases in the plasma membrane that may contribute to the calcium wave. The calcium influx channels may be opened in response to photostimulation, increasing the cytosolic concentration of calcium (Bonza et al. 2013). In order to completely inhibit the calcium wave, it may be necessary to block proton-calcium antiporters and calcium ATPases in the plasma membrane of these cells in addition to blocking the suspected voltage-gated ion channels. Future experiments can be performed to see if this is actually the case and that there are multiple sources of calcium involved in the calcium wave.

Presence of Voltage Gated Ion Channels

In order to tell if there are voltage-gated ion channels in the endoplasmic reticulum, *Arabidopsis* seedlings were incubated in different concentrations of gadolinium for approximately 55 minutes and before photostimulation. It has been shown that the minimum effective concentration of gadolinium to block a voltage-gated ion channel is10mM (Klüsener, 1995). Because of these findings, it was hypothesized that gadolinium would not be an effective inhibitor at lower concentrations and that the minimum effective dose would be 10mM of gadolinium.

A minimum effective dose for inhibition would explain why the 0.1mM and 1mM concentrations of gadolinium appeared to be ineffective at inhibiting the initial rise in cytosolic calcium levels following photostimulation when compared to the control values. The lack of inhibition may not be because the endoplasmic reticulum lacks voltage-gated ion channels, but because the dose was just too low to block the voltage gated ion channels. In contrast, the 10mM concentration of gadolinium did show a smaller increase in cytosolic calcium levels following photostimulation in comparison to the control values. The decrease in calcium levels supports the idea that gadolinium is capable of blocking voltage-gated ion channels, and that by blocking these channels; the release of calcium from the endoplasmic reticulum to the cytosol of the cell is inhibited. As previously discussed, the presence of an increase in cytosolic calcium levels seen with the 10mM concentration of gadolinium may be due to calcium influx through the plasma membrane, and calcium coming through the hydrogen-calcium antiporters and the calcium ATPases.

Future Experiments

It is possible that the rise in cytosolic calcium levels see with the 10mM concentration of gadolinium are due to calcium influx through the plasma membrane of the cell, as discussed previously. Other studies have shown that eosin Y is a known inhibitor of plasma membrane efflux of calcium and that external ATP in combination with L-glutamate can cause an increase in the levels of cytosolic calcium levels (Bonza et al, 2013). It is also known that there are calcium influx channels in the plasma membrane that are sensitive to gadolinium (Demidchik, 2002).

Future experiments that need to be performed should involve trying to inhibit the suspected voltage-gated ion channel in the endoplasmic reticulum, gadolinium sensitive plasma membrane calcium channels, and the calcium efflux channels and calcium ATPases in the plasma membrane. The overall goal would be to see if it is possible to see no net increase in cytosolic calcium in the cell in the presence of gadolinium and eosin Y. This would show that the source of the calcium in the calcium wave is from voltage gated ion channels in the endoplasmic reticulum, calcium channels in the plasma membrane, and from calcium influx across the plasma membrane. Another future experiment would be to see what effect the inhibitory gadolinium would have on the calcium level when in the presence of the stimulatory combination of external ATP and glutamate.

Summary

The inhibition of voltage-gated ion channels in the endoplasmic reticulum of *Arabidopsis thaliana* with the heavy metal, gadolinium, led to a decrease in the rise in cytosolic calcium

levels following photostimulation of the ER-chloroplast nexus. The lowest two concentrations of gadolinium, 0.1mM and 1mM, were shown to be ineffective at inhibiting the cytosolic calcium increase. However, the highest concentration, 10mM was shown to be an effective inhibitor, which is further supported by other studies that found 10mM gadolinium to be the minimum concentration of gadolinium effective at inhibiting voltage-gated ion channels. This implies not only that there are voltage-gated ion channels in the endoplasmic reticulum of these plants, but also that the voltage-gated ion channels contribute to the calcium wave. Future experiments can be done to try to inhibit other possible sources of calcium in the wave, such as the plasma membrane.

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