Effects of Gαq-PLCβ Pathway Activation on Cellular Ca^{2+} and Mitochondrial Activity

WPI

A Major Qualifying Project Report

Submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

In Partial Fulfillment for the

Degree of Bachelor of Science

in Biochemistry

On April 30th, 2021

Submitted By:

Emily Vail

WPI Advisor:

Prof. Suzanne F. Scarlata

Table of Contents

Abstract

The Gαq-PLCβ pathway is triggered when a neurotransmitter, hormone, or other signalling molecule binds to a G-protein coupled receptor located on the surface of the cell membrane. This results in a series of steps that lead to increased levels of cellular calcium ions. In this project, fluorescent sensor dyes and intrinsic fluorescence of NADH/NAD+ were used to show that increased levels of intracellular calcium ions after carbachol stimulation occur in parallel with increased mitochondrial activity.

Background

G-protein coupled receptors are a family of receptors that are crucially important in cell signalling and response to environmental changes. They react to a broad range of stimuli, and they trigger many different cellular responses. Their physiological roles in humans include receiving sensory input from sights, tastes, and smells, use in the endocrine system, binding to neurotransmitters in the brain, and more. Their ubiquity across most cells in the body and their specificity for each of their target signalling molecules makes them a common drug target. Over one third of prescription drugs approved by the FDA target G-protein coupled receptors (3). In the brain, they play a key part in behavior, mood, and brain function as they receive signals from different neurotransmitters. The Gαq-PLCβ pathway is a G-protein coupled receptor response pathway that cells use to respond to signaling from outside molecules. This particular pathway is activated when a specific hormone or neurotransmitter, such as carbachol, binds to a G-protein linked receptor on the cell membrane. This binding activates Gαq, which in turn activates PLCβ, which catalyzes hydrolysis of phosphatidylinositol 4,5, bisphosphate, which promotes Ca2+ release from the endoplasmic reticulum. The increase in cellular Ca2+ triggers different responses (4)

Environmental stress, including heat, hyperosmosis, chemical stress, and more, can result in the formation of protein-mRNA aggregates called stress granules. These are membraneless organelles that consist of aggregated mRNA and proteins. The main function of stress granules is to stop nonessential protein synthesis and conserve cell energy (4). In this way, resources can be conserved and the cell can survive the stressful environment because it is not wasting energy trying to grow, replicate, or perform specialized functions. Formation of the assembly and dissociation of stress granules is influenced by many different complex and intersecting regulatory processes, including signalling pathways responding to stimuli outside the cell. Stress granules have been linked to certain cancers and neurodegenerative diseases such as Alzheimer's disease (5).

Mitochondria are responsible for most of a cell's energy production in the form of ATP. This occurs through the electron transport chain, in which NADH and $FADH_2$ are oxidized to provide electrons that power the pumping of protons into the intermembrane space. This creates a proton gradient, which is used by ATP synthase to generate ATP from ADP and inorganic phosphate (6). Mitochondria are also sometimes used by the cell to store calcium ions, which, upon release from the mitochondria, can affect secondary signalling pathways and trigger cellular processes such as neurotransmitter release (7). Additionally, mitochondrial activity, especially in regards to ATP production, is closely related to regulation of the different stages of the cell life cycle (8). This becomes especially important to consider in the context of cancerous cells, which need a large supply of ATP to proliferate rapidly.

The Scarlata lab has previously found connections between the Gαq-PLCβ pathway and stress granule formation through their common component, PLCβ (4). The lab has also found evidence supporting a link between pathway activation and mitochondrial activity, which this research seeks to explore. Learning more about the complex interplay between cellular response to external signaling, the cell life cycle, stress granules, and mitochondrial activity will help to elucidate the mechanisms that govern multifaceted neurological conditions and diseases such as Alzheimer's, ALS, and cancer.

Methods

1. Cell culture & Differentiation

PC12 cells are a neuronal cell line developed from a pheochromocytoma tumor in the adrenal medulla of rats (2). For all of the experiments in this project, cells were cultured with Gibco Dulbecco's Modified Eagle Medium (DMEM), a standard mammalian cell medium. For each experiment, new dishes of cells were split from a dish of high confluency and 24 hours were allowed to pass for the cells to grow. After 24 hours, cells were differentiated by placing cells in differentiation media and adding 1 μM nerve growth factor (NGF). 24 hours were again allowed to pass before staining or stressing the newly differentiated cells.

2. Calcium Green & MitoTracker stains

Staining with Calcium Green took place 24 hours after differentiation. Each dish of cells was aspirated and 1 mL of Hank's Buffered Saline Solution (HBSS) was added. Then, 8 μL of Calcium Green dye suspended in dimethyl sulfoxide (DMSO) was added to each dish and the dishes were incubated in the dark at 37 ℃ for approximately 30 minutes. After incubation, each dish was aspirated, washed with phosphate-buffered saline (PBS) and then either left to incubate for 24 hours or prepared for overnight stress (See Methods, Section 3). 24 hours after cells were stained with Calcium Green, they were stained with MitoTracker Red. The MitoTracker Red dye was suspended in 92 μL of DMSO. 2 μL of this dye solution was then added to 1 mL of PBS. Each dish was aspirated, and then 2 mL of HBSS and 100 μL of diluted dye solution added. The dishes were then incubated in the dark at 37 ℃ for 40 minutes, aspirated and washed with PBS, and then prepared for imaging.

3. Stress conditions

1 mM carbachol was used as the primary chemical stressor in both experiments for this project. In the [Ca^{2+}] & Mitochondrial Activity experiment, 30% hydrogen peroxide was also used as a control to show that calcium levels and mitochondrial activity reacted together to other types of stress through the Gαq-PLCβ pathway. For this experiment, four different stress conditions were tested in addition to two controls according to the table below. Overnight stress conditions were constructed by adding 2 μM to the dishes' media and letting them sit in the incubator for 24 hours. Acute stress conditions were obtained by directly pipetting the appropriate amount of carbachol or hydrogen peroxide over cells being imaged in the dish.

Condition	Concentration
Overnight Carbachol	2 μM overnight, 5 μM acute
Carbachol	5 μM acute
Hydrogen Peroxide	100 μM acute
Hydrogen Peroxide	150 μM acute
Overnight Carbachol Control	1 μM overnight
No Stress Control	N/A

For the fluorescence lifetime imaging microscopy (FLIM) experiment, carbachol stress was all timed exposure ranging from carbachol concentrations of 2-5 μM and duration from 10 minutes to 48 hours. There were two additional conditions with two hours of stress recovery time between stressing and imaging. For the recovery period, cells were aspirated, washed with PBS, and given fresh media with no carbachol.

4. FLIM

For the FLIM experiment, cells were not stained before imaging. Some dishes of cells received 24 or 48 hours of carbachol stress prior to imaging, and the rest of the stress occurred directly before imaging. To image, dishes were aspirated, washed, and 2 mL of HBSS was added. The experiment utilized the ISS Alba FLIM 2-photon confocal microscope with a 60x water immersion objective that had been calibrated with Atto 435 and Alexa 488 dyes. During imaging, the excitation wavelength for each dish was 740 nm and the FastFlim setting was used.

5. $[Ca^{2+}]$ & Mitochondrial Activity

For the Calcium Green and MitoTracker experiment, after each dish was stained with MitoTracker red, it was aspirated, washed with PBS to remove all traces of dye outside the cells, and 2 mL of HBSS was added. Cells on each dish were imaged for 9 frames using the Zeiss LSM 510 meta confocal microscope using a 40x water immersion objective and excitation wavelengths of 543 nm and 633 nm. Controls were imaged continuously without pausing. For acute stress conditions, imaging was paused after 9 frames and resumed at the same time as being acutely stressed.

6. Analysis

For FLIM, a Phasor plot was obtained for each cell after imaging. The plot was set to smooth rank 2 and thresholded. Phasor data was then used to collect fluorescence lifetime, and the results were plotted using SigmaPlot. Eight cells were used for each condition.

To calculate relative intensity of the Calcium Green and MitoTracker Red stains, a ROI was selected for each cell in the imaging software, and the table of intensity over time from the software was copied into an analysis spreadsheet. Average normalized intensity

over time was calculated for the red and green channels for each cell and plotted using SigmaPlot.

Results and Discussion

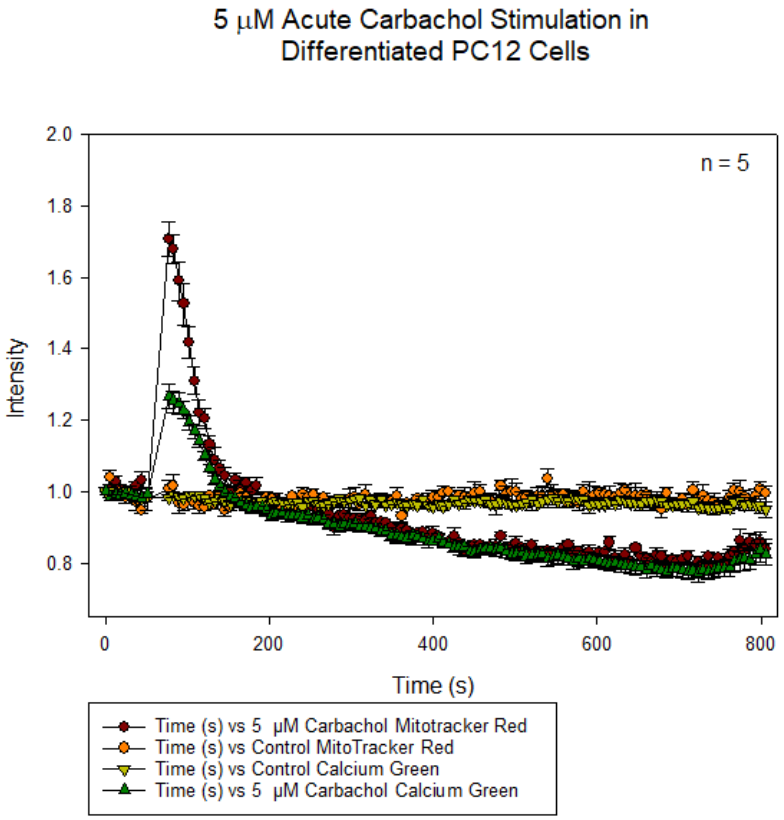

Figure 1: After stimulation, mitochondrial activity and Ca^{2+} levels immediately increased, and then over time fell below baseline levels

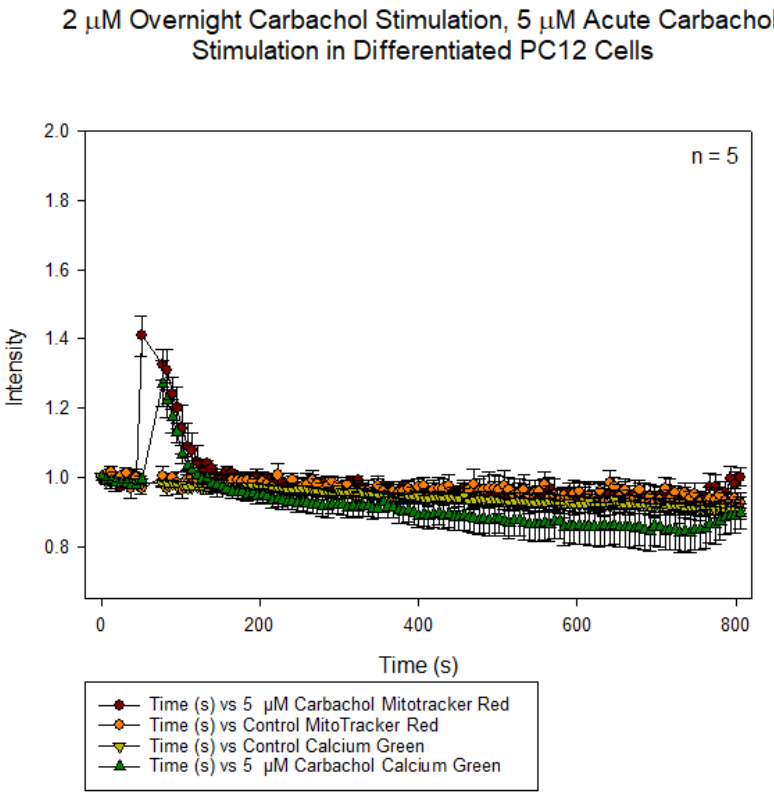

Figure 2: In cells which had already been stressed overnight, a similar trend to just acute stress is observed, however it is less pronounced.

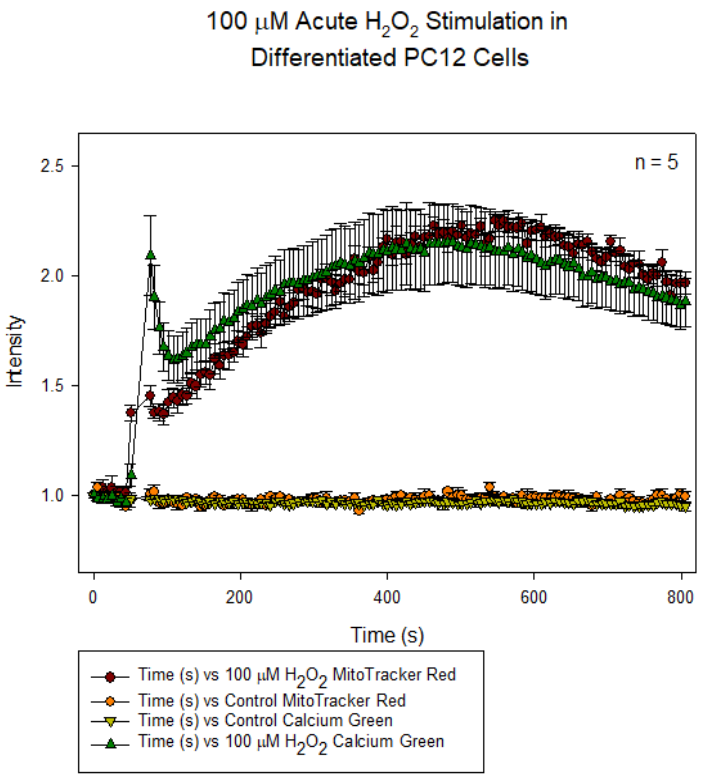

Figure 3: With hydrogen peroxide stress, there is an initial spike in mitochondrial activity and Ca^{2+} which drops, then slowly increases and decreases

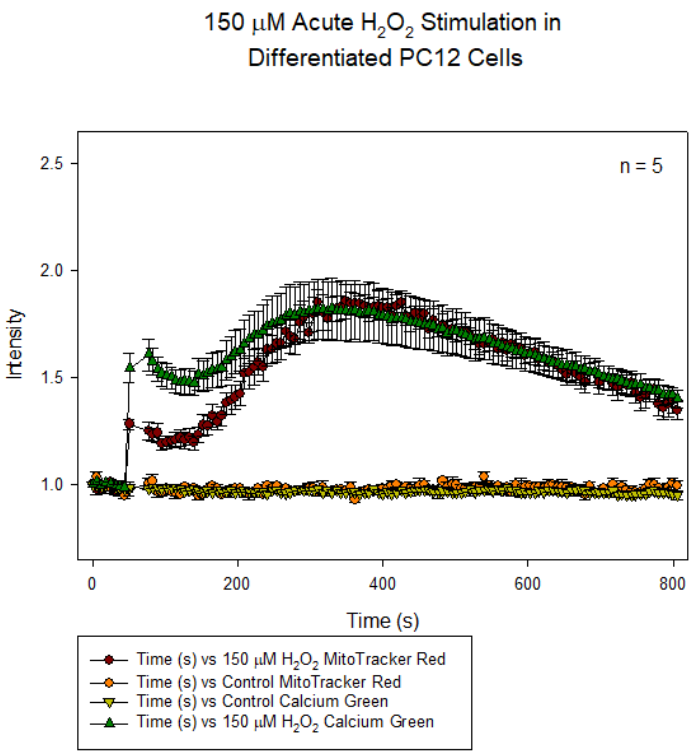

Figure 4: With a larger concentration of hydrogen peroxide, a similar but less pronounced trend is observed.

The fluorescent sensor MitoTracker was used to monitor cell mitochondria activity under stress and compared to total cell Ca^{2+} responses. As shown in figures 1 and 2, the trends for both responses closely match one another for initial stimulation, but the mitochondria response was dampened with extended stimulation. These results show that increased Ca^{2+} from Gαq-PLCβ pathway activation is paralleled in the mitochondria, but with extended activation, mitochondria do not completely recover.

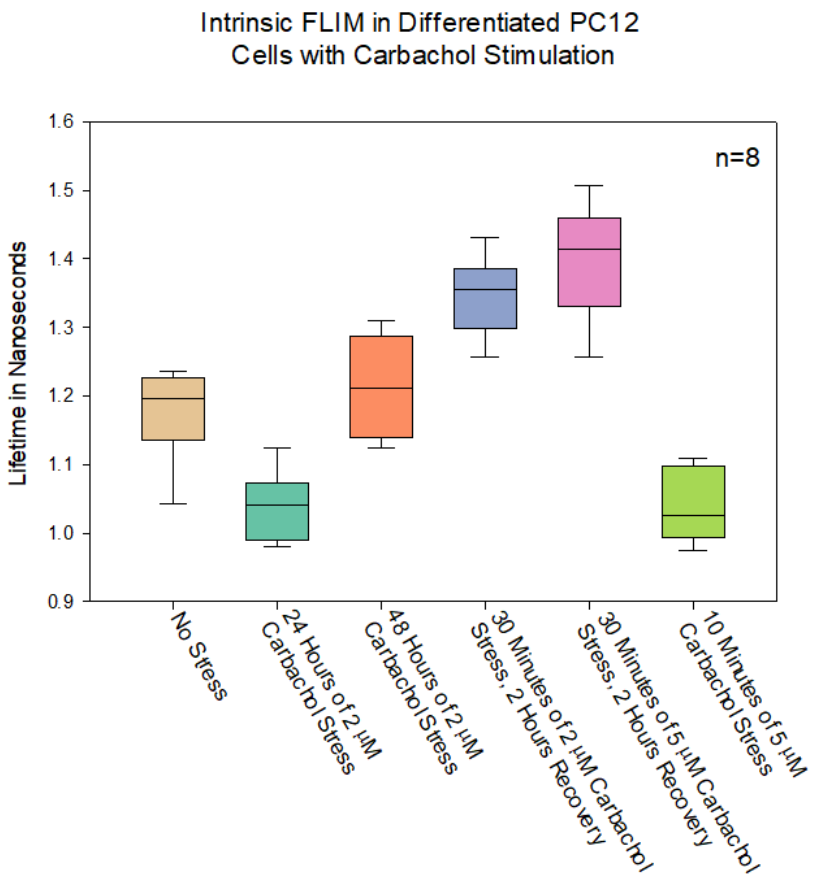

Figure 5: Each stress condition displays varying levels of fluorescence activity

The fluorescence lifetime imaging microscopy (FLIM) experiment measured intrinsic fluorescence mainly from NADH, which changes upon oxidation to NAD+ in the electron transport chain which generates ATP. The relative NAD+/NADH levels as detected by FLIM indicate oxidative stress in the mitochondria. Increased exposure to carbachol and increased recovery time from stress both seem to result in greater oxidative stress in the mitochondria. The greatest oxidative stress observed was found in the sample which received 30 minutes of 5 µM carbachol followed by two hours of recovery time. Except for the 48 Hours of 2 µM Carbachol Stress condition, there were statistically significant differences between lifetimes for each stress condition compared to the control. P-values for each condition were calculated using SigmaPlot to conduct equal variance assumed t-tests according to the table below.

Condition	24 Hours of 2 μM Carbachol	48 Hours of 2 μM Carbachol	30 Min. of 2 μM Carbachol	30 Min. of 5 μM Carbachol	10 Min. of 5 μM Carbachol
P-Value	0.000298	0.310	0.0000679	0.0000444	0.000360

The results from both experiments indicate that activation of the Gαq-PLCβ pathway with a signalling molecule such as carbachol has very similar effects on both the concentration of calcium ions in the cell and the activity of the mitochondria. This activation results in increased oxidative stress in the mitochondria, which is more intense with greater length of exposure to stress and greater concentrations of carbachol. Throughout all experiments and stress conditions, the only condition to result in significant cell death was 150 μM of hydrogen peroxide.

Conclusion

There is an evident link between the calcium ions that flood a cell after chemical stress and the accompanying increase in mitochondrial activity, as shown by increased Calcium Green and MitoTracker fluorescence after stimulation with carbachol and hydrogen peroxide. Activation of the Gαq-PLCβ pathway plays a major role in this process, although it is as of yet unclear how different stimuli achieve different patterns of cellular effects. Recovery from stress also seems to differ between types of chemical stress, as cells from the hydrogen peroxide trials never returned to baseline levels of calcium and mitochondrial activity. It is also as of yet unknown how this increase in activity affects energy production and consumption in the cell. In addition to the increased mitochondrial activity observed after stimulation, there is also increased oxidative stress in the mitochondria following carbachol stimulation. It is not yet known if the influx of calcium plays a direct role in activation of the mitochondria, or if they are simply different resulting processes from the same initial activation. Due to the similarity in their trend as shown by the fluorescent sensor experiments, it is likely that there is a relationship linking the two processes.

For further exploration into this subject, a future experiment could transfect PC12 cells with an ATP sensor plasmid that will tag cellular ATP with green fluorescent protein. Then, endogenous ATP levels could be determined and compared to ATP levels after stimulation. This will help to determine if energy production in the cell increases as a result of Gαq-PLCβ pathway activation, and to what extent. It may also be helpful in determining exactly what mitochondrial components are being activated by the stress response pathway, such as ATP synthase (ATP5f1b).

Acknowledgements

First and foremost, I would like to thank Professor Suzanne Scarlata for her guidance throughout this project and for allowing me the opportunity to research such an interesting subject. I would also like to thank Lela Jackson for her support over the past few months, and for everything she has taught me about research and lab skills. Having the community of everybody in the Scarlata lab was a valuable resource, and I am very grateful to have worked with them as well. Finally, I would like to thank the faculty of the Chemistry and Biochemistry department for continuously sparking my passion for biochemistry and helping me reach the path I am on today.

Bibliography

1. Blacker, T., Duchen, M. (2016). Investigating mitochondrial redox state using NADH and NADPH autofluorescence. *Free Radical Biology and Medicine*, *100*. 53-65. Doi: https://doi.org/10.1016/j.freeradbiomed.2016.08.010
2. Wiatrak, B., Kubis-Kubiak, A., Piwowar, A., Barg, E. (2020). PC12 Cell Line: Cell Types, Coating of Culture Vessels, Differentiation and Other Culture Conditions. *Cells*, *9* (4). doi: https://doi.org/10.3390/cells9040958
3. Sutkeviciute, I., & Vilardaga, J. P. (2020). Structural insights into emergent signaling modes of G protein-coupled receptors. *The Journal of biological chemistry*, *295*(33), 11626–11642. doi: https://doi.org/10.1074/jbc.REV120.009348
4. Qifti, A., Jackson, L., Singla, A., Garwain, O., & Scarlata, S.. *bioRxiv* 521369. doi: https://doi.org/10.1101/521369
5. Protter, D., & Parker, R. (2016). Principles and Properties of Stress Granules. *Trends in cell biology*, *26*(9), 668–679. Doi: https://doi.org/10.1016/j.tcb.2016.05.004
6. Fernie, A.R., Carrari, F., & Sweetlove, L.J.. (2004). Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport. *Curr Opin Plant Biol.* *7*(3). 254-61. doi: https://doi.org/10.1016/j.pbi.2004.03.007
7. Santulli, G., Pagano, G., Sardu, C., Xie, W., Reiken, S., D'Ascia, S.L., Cannone, M., Marziliano, N., Trimarco, B., Guise, T.A., Lacampagne, A., & Marks, A.R. (2015). Calcium release channel RyR2 regulates insulin release and glucose homeostasis. *The Journal of Clinical Investigation. 125* (5). 1968–1978. Doi: https://doi.org/10.1172/JCI79273
8. Sweet, S., Singh, G. (1999). Changes in mitochondrial mass, membrane potential, and cellular adenosine triphosphate content during the cell cycle of human leukemic (HL-60) cells. *Journal of Cellular Physiology*, *180* (1). 91–96. Doi: https://doi.org/10.1002/(sici)1097-4652(199907)180:1%3C91::aid-jcp10%3E3.0.co;2-6