Investigating the Effects of Salt Stress on the Morphology of PC12 Cells

WPI

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ABSTRACT

The PLC□1 pathway in the PC12 cell line is responsible for the production of calcium, which is used as a form of signaling between cells. This type of chemical communication between cells is made possible by long neurites that extend from the soma of one cell to the dendrites of another. When the gap of the synapse across which chemical signals must pass has been decreased, successful reception of said signals becomes much more feasible. If these neurites were made to retract away from other cells, signaling would become less viable and cellular growth rates would decline. To investigate a potential cause of neurite retraction, PC12 cells were subjected to 0.22M NaCl stress for either 3min or 6min. In half of the trials, the cells were provided with recovery time in ideal incubation conditions after the NaCl had been removed. Upon imaging using immunofluorescence microscopy, the most extreme instances of neurite retraction were observed from the 3min stress condition without a recovery period. Additionally, neurites were not perceived to be in a retracted state whenever they were given a recovery period following NaCl stress. Understanding more about neurite retraction is critical, since they are directly related to the pathway that plays a significant role in processes as paramount as memory and cognition.

BACKGROUND

Phospholipase C□ (PLC□) is a protein complex that is monumental in the conversion of extracellular signals to intracellular calcium (Rennie et al., 2022). To expand upon this, a ligand such as acetylcholine, serotonin, or histamine will first bind to a G protein-coupled receptor (GPCR) that is embedded in the membrane of a cell. This causes the associated G protein known as Gαq to become activated and bound to the energy-storage molecule guanosine triphosphate (GTP), which will then allow Gαq to bind PLC□. Upon the association of these components, phosphatidylinositol (4,5)-bisphosphate (PIP_2) can then be hydrolyzed to yield inositol (1,4,5)-triphosphate (IP_3). IP_3 is then able to open calcium channels by diffusing through the endoplasmic reticulum (Rennie et al., 2022). There are four isozymes of PLC□, with PLC□1 being the most sensitive when it comes to the conversion of a Gαq signal to increased intracellular calcium. The aforementioned mechanism involving PLC□1 is shown in FIGURE 1.

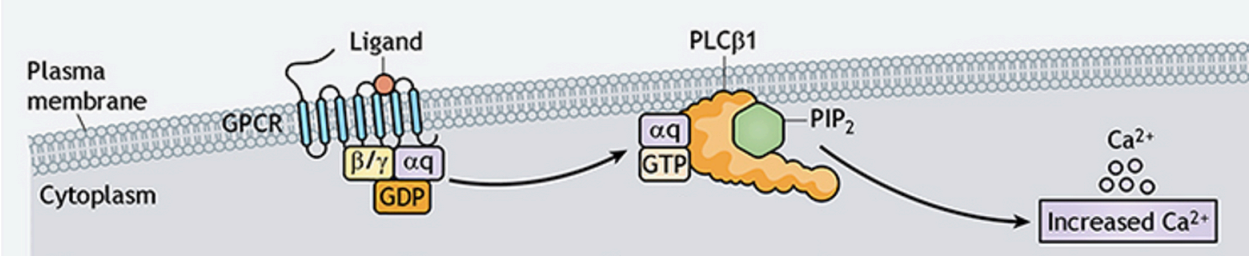

FIGURE 1. **PLC□1 Involvement in Intracellular Calcium Production.** An extracellular signal in the form of a ligand binds to a GPCR, which activates Gαq so it can bind to PLC□1 and convert PIP_2 to IP_3. IP_3 is then able to open calcium channels in the cell (Rennie et al., 2022).

The release of calcium into the cytoplasm of a cell is important because this cation is involved in a variety of necessary processes such as muscle contraction and gene transcription (Bootman, 2012). Additionally, PLC□1 is important because it is associated with neuronal development, learning, and memory (Rennie et al., 2022). Because of these extremely relevant associations, it is evident that cells containing PLC□1 warrant further study. One type of cell line that can be used for PLC□1 manipulation is the PC12 strain, derived from rat pheochromocytoma cells (Wiatrak et al., 2020). These cells are attachment-dependent, and can be made to differentiate using nerve growth factor (NGF). When PC12 cells are differentiated, PLC□1 production is increased significantly (Rennie et al., 2022). FIGURE 2 shows the difference between non-differentiated PC12 cells and differentiated PC12 cells.

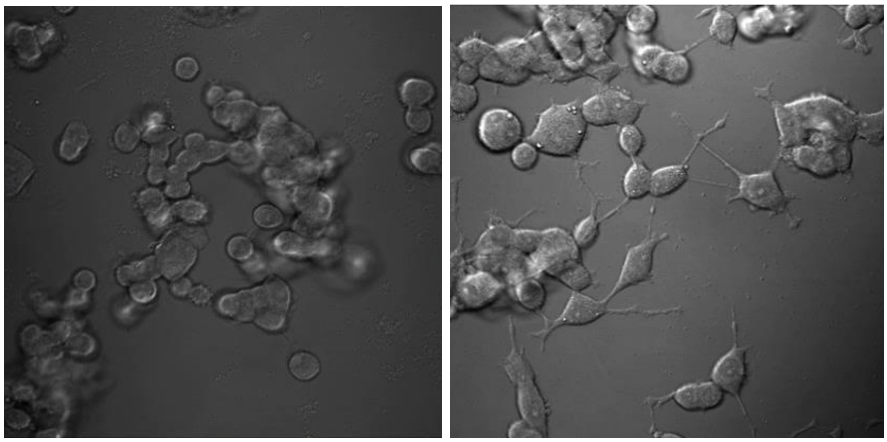

FIGURE 2. **Non-differentiated (Left) and Differentiated (Right) PC12 Cells.** Cellular communication through signaling is promoted in the right image due to the presence of neurites connecting cells that are far apart (Garwain & Scarlata, 2016).

In utilizing differentiated PC12 cells to investigate PLC□1, there are many different routes for research. It has already been established that extended duration of acetylcholine exposure causes the neurites between cells to become severed and to retract due to the production of calcium by the PLC□1 pathway, which is disadvantageous for the efficient communication between cells that is so critical for their growth (Rennie et al., 2022). It has also been proposed that neurite retraction occurs because of mechanical stress imparted by ion flux, resulting in altered membrane tension. This neurite retraction is shown in FIGURE 3.

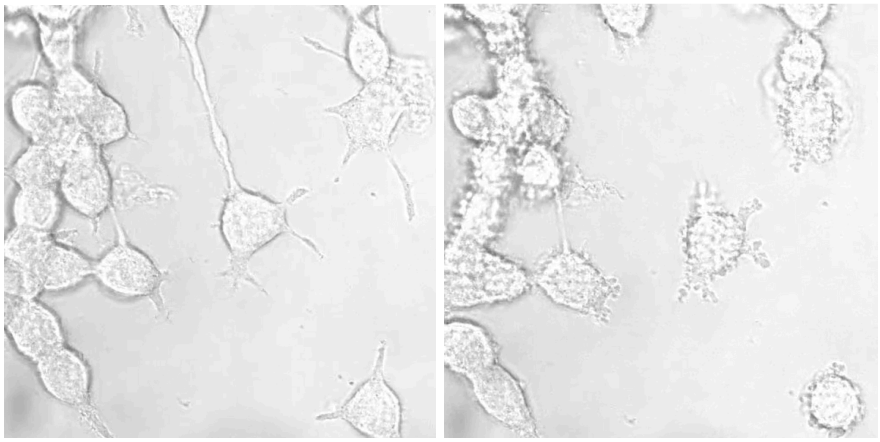

FIGURE 3. **Neurites Between Cells Before (Left) and After (Right) Retraction Following Extended Acetylcholine Exposure.** Cellular communication through signaling is promoted in the left image with the intact neurites, and it is evident that the recoiling of the neurites following their retraction is not ideal for signaling (Pearce et al., 2020).

Many cells are only able to communicate by releasing signals that can travel short distances to their respective receptors; when these receptors are moved much further away–which refers to the retraction of neurites–communication through signaling is no longer

feasible. This may result in impeded cognitive processes, which is a cause for concern. This raises the question: what else causes the neurites between cells to retract so dramatically? In order to investigate this, PC12 cells were subjected to salt stress in the form of NaCl, which was meant to supply ions that would flow by and subsequently alter membrane tension. NaCl was used over other salts because it has already been established that glycerol and $MgCl_2$ do not provoke neurite retraction at all, and KCl only does so in exceedingly rare instances (Pearce et al., 2020).

Upon NaCl exposure, it was observed that the neurites retracted to the soma in a similar manner to when they were exposed to acetylcholine for an excessive amount of time. Because of the movement caused by this retraction and subsequent recoiling, the aggregated neurites assumed a fluid consistency in comparison to the more tightly-packed cellular contents, as well as the extracellular space. Staining with a fluorescent dye known as Laurdan–which adheres to cellular components of a more fluid consistency (Sanchez et al., 2020)–indicated the successful retraction and phase change of the neurites due to the NaCl exposure.

METHODS

Cell Culture and Differentiation

The PC12 cells were cultured in 100mm Falcon Petri Dishes using Dulbecco's Modified Eagle Medium (DMEM) containing 4.5g/L D-glucose and L-glutamine. To split the cells and promote uncrowded growth, the complete media was aspirated using vacuum tubing, the pertinent plate was washed twice with 5mL Dulbecco's Phosphate Buffered Saline (PBS), and 2mL <0.05% trypsin was added to cause the cells to detach from the bottom of the plate. After about 5min, 3mL DMEM was added to the plate. The cells were then mixed into the solution using a micropipette, and a certain volume was transferred to a new plate. Finally, DMEM was added to bring the resulting volume up to 10mL.

Once the plate had reached over 70% cellular confluence, its cells were split into five 35mm Falcon Petri Dishes. 85uL of the detrypsinized solution was placed in each of the dishes, and DMEM was added to bring the total volume of each up to 2mL. These plates, like the stock, were incubated at 35°C with 5% CO_2 for 24hrs. Following this, cellular differentiation was induced. The media for differentiation was prepared by combining 10uL 100ug/uL NGF with 9.9mL DMEM containing the same aforementioned components. Following its preparation, 2mL of this differentiation media was added to each of the dishes after the original solution was aspirated and the plates were washed with PBS. The dishes were then placed in the incubator for 48hrs to promote ideal growth and differentiation.

NaCl Stress

Once the cells were given ample time to differentiate, the different experimental conditions were imparted upon them. The five conditions were as follows: a non-differentiated control dish (ND), a dish subjected to 0.22M NaCl stress for 3min followed by a 15min recovery in the incubator (3R), a dish subjected to 0.22M NaCl stress for 3min with no recovery period (3S), a dish subjected to 0.22M NaCl stress for 6min followed by a 15min recovery in the incubator (6R), and a dish subjected to 0.22M NaCl stress for 6min with no recovery period (6S). To prepare the 3R, 3S, 6R, and 6S dishes for stressing, their differentiation media was aspirated out, they were washed twice with 2mL PBS, and 500uL PBS was added to each. Then, 500uL 0.22M NaCl was added to each dish, and the additions were timed precisely so that the salt was removed after either 3min or 6min.

Following the removal of the NaCl from the 3R and 6R dishes through aspiration, the plates were washed twice with 2mL PBS, 2mL complete media was added, and the dishes were placed in the incubator for 15min. Following this period of rest, the solution from all five dishes was aspirated and the plates were washed twice with 2mL PBS. Then, the cells were fixed using 1mL of a solution made of 500uL 3.7% formaldehyde and 4.5mL PBS. After 20min, the solution was removed from the dishes and they were each washed three times with 2mL PBS. Finally, 1mL PBS was added to the dishes and they were placed in the cold room–which maintains a temperature of 4°C–until immediately prior to staining and imaging.

Laurdan Staining

Right before the cells were to be imaged using the ZEISS Confocal Laser Scanning Microscope 510 (LSM510), they were stained with Laurdan. This was done by first aspirating the existing PBS in each dish, washing three times with 2mL PBS, and leaving 1mL PBS in each. To this, 40uL Laurdan was added to each dish and allowed to stain the cells for 15min. However, it is important to note that a 150 Falcon Petri Dish was covered in aluminum foil, and each of the five 35mm dishes were placed inside while the Laurdan was acting. This is because Laurdan is light-sensitive, and its staining properties will be greatly diminished if they are exposed to light for an excessive amount of time. After the Laurdan had stained the cells for 15min under the cover of darkness, the solution in each dish was removed, and each plate was once again washed three times with 2mL PBS. Finally, 1mL PBS was left in each dish, and they were ready for imaging.

Cell Imaging and Analysis

The PC12 cells in each of the five dishes were imaged using three different light fields of the ZEISS LSM510 under 63x fluorescence microscopy at a 270nm excitation. Images of the cells were captured in the bright field, green field (picking up on green fluorescent protein (GFP) localization), and the red field (picking up on Laurdan localization). Stacked images of these three fields were also captured. ImageJ software was utilized for the adjustment and analysis of the quality of these images. The bright field was used in order to view the recoiled nature of PC12 neurites following NaCl stress. The green field was used to confirm the existence of live cells over debris, since GFP adheres to the cytoplasm in a manner that accentuates the intact cell membrane as well as the nucleus. The red field was used to match any brightly-colored substances to the recoiled neurites from the bright field. Since Laurdan adheres to more fluid substances, this had the purpose of confirming the presence of recoiled neurites.

RESULTS

In analyzing the images of the PC12 cells under the bright field, it was immediately evident that the addition of NaCl was causing neurites to retract. This neurite retraction and subsequent recoiling is presented in FIGURE 4.

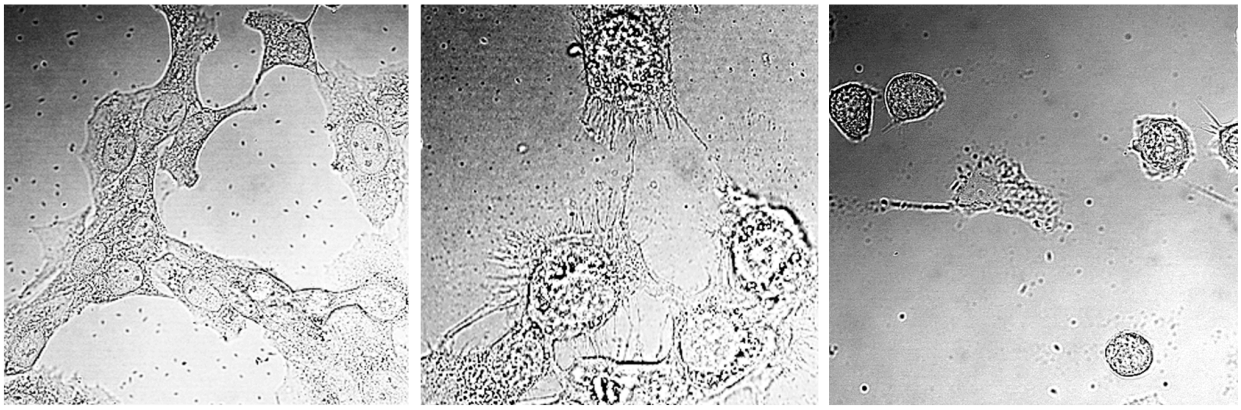

FIGURE 4. **Neurites Between PC12 Cells Before (Left), During (Middle), and After (Right) Retraction from Extended NaCl Exposure.** The left image shows the plethora of thick neurites connecting cells, the middle image reveals the prevalence of thinner neurites between cells, while the right image highlights the lack of intact neurites and the existence of recoiled neurites.

When focusing solely on the images captured from the non-differentiated control (ND) condition, it is once again evident that the neurites connecting cells are thick and therefore reinforced for more efficient communication through signaling. The green field was successful in confirming that the captured images contained live cells, and the complete lack of bright red areas in the red field evidenced the absence of recoiled, fluid neurites. All of this can be viewed in FIGURE 5, which also provides a stacked image from each of the three aforementioned fields.

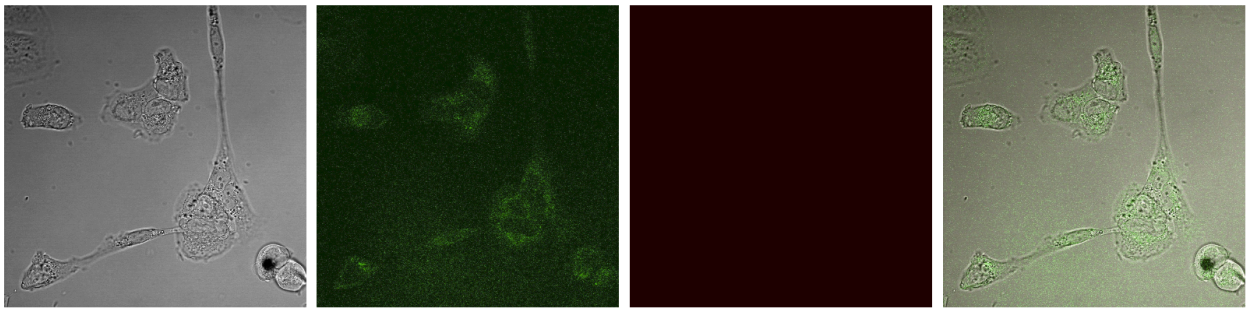

FIGURE 5. **Non-differentiated PC12 Cells Viewed in the Bright Field (Leftmost), GFP Field (Left/Middle), Red Field (Right/Middle), and All Fields Combined (Rightmost).** These images were acquired using the ZEISS LSM510. Since these cells represent the control for the experiment, it makes sense that the red field is blank.

Moving on to one of the experimental conditions where NaCl stress took place, the dish subjected to 3min stress without recovery (3S) was clearly indicative of the presence of retracted neurites that had recoiled and assumed a more fluid state. This was evidenced by both the direct appearance of recoiled neurites in the bright field, as well as the bright red color on the edges of the cells in the red field. Once again, the green field was useful in the confirmation of live cells. All of this can be viewed in FIGURE 6, which also provides a stacked image from three of the aforementioned fields.

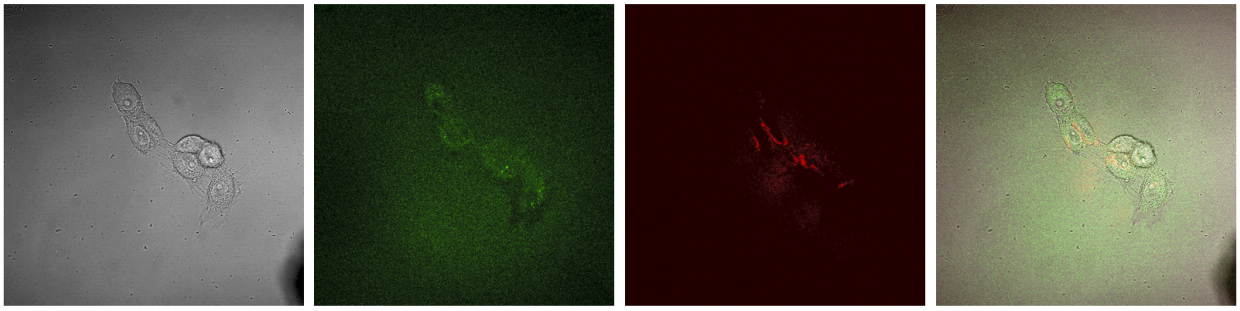

FIGURE 6. **3S PC12 Cells Viewed in the Bright Field (Leftmost), GFP Field (Left/Middle), Red Field (Right/Middle), and All Fields Combined (Rightmost).** These images were acquired using the ZEISS LSM510. The red field is particularly significant because it shows that the Laurdan stain has adhered to the edges of the cells where the neurites have recoiled and assumed a more fluid consistency.

The results pertaining to the dish subjected to 6min stress without recovery (6S) were very similar to those of the 3S condition, the main difference being that the retracted neurites had not transitioned into a fluid phase as much as the neurites in the 3S condition. This is evidenced by the fact that far less Laurdan staining was picked up in the red field. However, the bright field still shows that neurites have recoiled, and the green field confirms the fact the cells are alive. All of this can be viewed in FIGURE 7, which also provides a stacked image from three of the aforementioned fields.

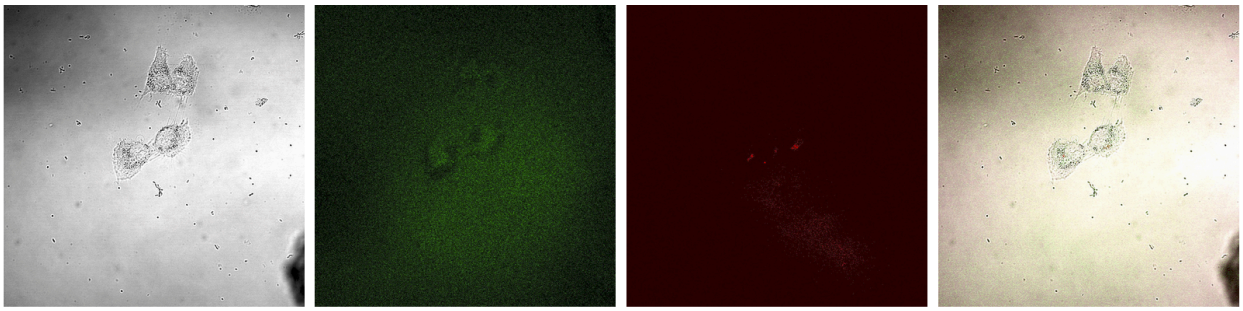

FIGURE 7. **6S PC12 Cells Viewed in the Bright Field (Leftmost), GFP Field (Left/Middle), Red Field (Right/Middle), and All Fields Combined (Rightmost).** These images were acquired using the ZEISS LSM510. The red from the red field is not as striking as in the 3S

condition in FIGURE 5, but its subtle appearance indicates that the recoiled fluid neurites are still present.

While it is true that the neurites in the dish subjected to 3min stress with recovery in the incubator (3R) have thinned out in comparison to those in the ND condition due to the NaCl stress, they did not appear to be retracted upon imaging. This was evidenced directly by the bright field, and indirectly by the red field due to the complete lack of visible Laurdan staining. The green field was once again used to confirm the live status of the cells. All of this can be viewed in FIGURE 8, which also provides a stacked image from three of the aforementioned fields.

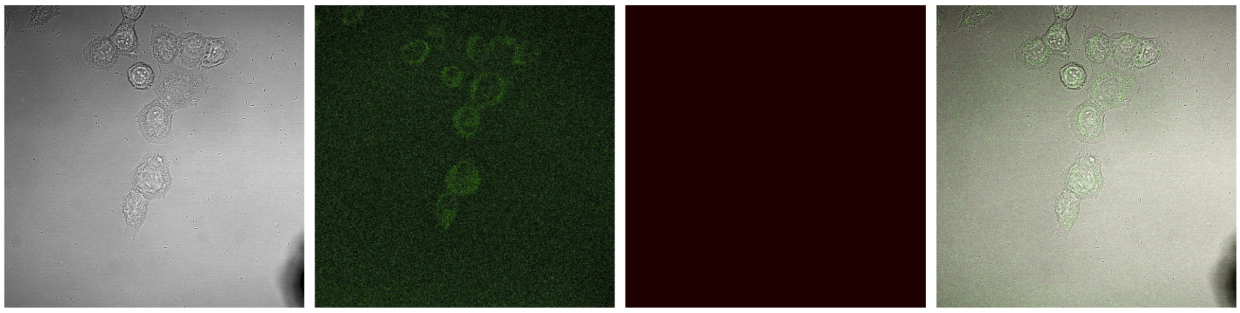

FIGURE 8. **3R PC12 Cells Viewed in the Bright Field (Leftmost), GFP Field (Left/Middle), Red Field (Right/Middle), and All Fields Combined (Rightmost).** These images were acquired using the ZEISS LSM510. The absence of red light in the red field indicates that there has been no recoiling of neurites or transformation into a fluid phase.

Similarly to the 3R condition, the neurites in the dish subjected to 6min of stress with recovery in the incubator (6R) appeared to be very thin in the bright field. Equally similar, the absence of visible Laurdan staining in the red field indicated that the neurites did not recoil and assume a fluid phase. Still, it should be noted that the cells are still alive and well, which was seen in the green field. All of this can be viewed in FIGURE 9, which also provides a stacked image from three of the aforementioned fields.

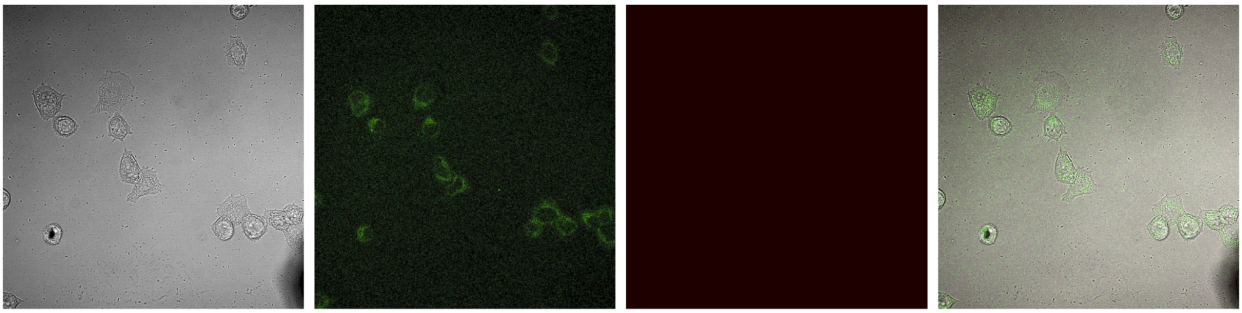

FIGURE 9. **6R PC12 Cells Viewed in the Bright Field (Leftmost), GFP Field (Left/Middle), Red Field (Right/Middle), and All Fields Combined (Rightmost).** These images were acquired using the ZEISS LSM510. As in the instance of the 3R condition in FIGURE 8, the

absence of red light in the red field indicates that there has been no recoiling of neurites or transformation into a fluid phase.

DISCUSSION

Since the PLC□1 pathway has such a significant involvement in cognition, memory, and neuronal development, it is of the utmost importance to learn more about the factors that both promote and inhibit this pathway. In a normally-functioning PLC□1 pathway, calcium will be released in a manageable quantity. However, there is now evidence to suggest that both the extended presence of acetylcholine and the extended exposure of PC12 cells to NaCl cause the PLC□1 pathway to produce of a surplus of intracellular calcium due to the prolonged opening of the respective channels in the membrane, which in turn results in the retraction and subsequent recoiling of neurites (Trump et al., 1980). Neurite retraction is not ideal because it prevents effective signaling between cells, resulting in the diminished occurrence of healthy cellular growth (Sainath & Gallo, 2014). If these PC12 cells are unable to adequately send and receive chemical signals due to severed neurites, then it follows that cognition and other associated processes may be severely impaired as a result. Because of these implications, the acquired results pertaining to the different NaCl stress conditions are highly relevant.

Before delving into the discussion of the results pertaining to the PC12 cells that were subjected to NaCl stress, the usefulness of the ND condition must first be addressed. The acquired images from this condition served as an excellent baseline for the appearance of normal PC12 cells. The prevalence and thickness of the neurites between the cells was a common finding between each of the images from this condition, as was the lack of Laurdan adherence to the cells in the red field. The aggregation of Laurdan staining is indicative of cytoskeletal protrusions assuming a more fluid state in contrast to tighter membrane packing, so it is not odd to perceive its absence in the red field since the neurites did not retract in this condition.

Pertaining to the two stress conditions without recovery periods, the acquired images were indicative of successful neurite retraction. The most extreme instance of this was found for the PC12 cells in the 3S condition. It was frequently observed that the PC12 cells in the 6S condition maintained neurites that had drastically thinned out, rather than being completely severed as in the 3S condition. However, there were still instances of neurite retraction for this condition. While this is still problematic for cell signaling, it is not as critical an issue as the complete recoiling of neurites to the soma. While one may think that the more lengthy exposure of PC12 cells to NaCl in the 6S condition would bring about more retraction than in the 3S condition, the opposite was observed. This could be because in the additional time afforded by the 6S experimental condition, the cells were able to begin their neurite regeneration process. Following the recoiling of neurites, calcium levels will eventually become normal again so that the intact neurites are regenerated. It is likely that there was simply no time for this regeneration to occur in the 3S condition.

Further, it is highly probable that promoted neurite regeneration is the reasoning behind the intact neurites in the 3R and 6R conditions. For each acquired image pertaining to these two conditions, there was no visible aggregation of Laurdan staining in the red field. These cells were much more similar in appearance to the ND condition than to either the 3S or the 6S condition.

Just like the images from the ND condition, the red field appeared completely black. It makes sense that this occurred, since the afforded time in ideal recovery conditions for growth would have promoted the regeneration of neurites even more so than in the 6S condition. However, it is important to note that there were not any striking differences between the appearances of neurites in the 3R and the 6R conditions. This is likely due to the speed of regeneration in the incubator being much faster than regeneration during NaCl stress, which would render any neurite growth prior to incubation incredibly minute in comparison to growth taking place during incubation.

CONCLUSIONS AND RECOMMENDATIONS

To summarize, the addition of NaCl to differentiated PC12 cells causes neurite retraction due to mechanical stress on the membrane imparted by Na^+ flux, triggering a defense mechanism that shrinks the spindled protrusions back into the soma. The 3S condition evidences the retraction and recoiling of neurites to the greatest extent, and the resulting fluid consistency of the neurites is evident from the visible Laurdan staining in the red field. A recovery period in ideal incubation conditions after NaCl stress likely prompts neurite regeneration back to their original state, since there is no visible Laurdan staining to indicate recoiling.

The implications of the aforementioned results elucidate the urgency with which the altered action of the PLC□1 pathway and subsequent morphological changes to the PC12 cell membrane due to NaCl stress should be further examined. The plethora of severed neurites between cells resulting from this stress could be an indication of potential cognitive impairment in the host organism. If further research were to be conducted in this realm, then more would undoubtedly be discovered about how to avoid this tragic fate in various organisms. The vast majority of the current publications relating to this topic are focused on the impact of NaCl on PC12 mitochondria rather than neurites, and additional experimentation pertaining to neurite retraction would be extremely useful (Hamilton & Heckathorn, 2001). Additional experimentation would serve to either confirm any existing postulates or suggest new ideas about the underlying mechanisms of PC12 neurite retraction. A good place to start–in terms of future experimentation–would be to record a series of time-lapses including the time during and after subjecting the cells to NaCl stress; this would allow for the acquisition of videos detailing complete neurite retraction and recoiling in real time. This experimental design would theoretically yield more thorough data than the experiment described by this paper, which only captured still images of the cells at a handful of different points in time. Experimental conditions to alter during the time-lapses include NaCl concentration, Laurdan concentration, time subjected to stress, and afforded recovery time. These newly-proposed experiments may serve to yield valuable results, so that new conclusions can be made regarding the nature of neurite retraction in PC12 cells. These conclusions may very well go on to prevent a decline in cognition, memory, and other functions in living organisms all over the planet, including people.

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