The Effects of Stretch on Intracellular Ca^{2+} Signaling in Cocultured Neuronal and Smooth Muscle Cells

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Abstract

The purpose of this project is to better understand how differentiated smooth muscle (WKO-3M22) and neuronal cells (PC12) vary their calcium signaling response with regards to repeated physical stress, and how the formation of neuromuscular junctions (NMJs) between the two cell types affects this response. Ca^{2+} signaling in cells is largely mediated by the protein $G\alpha_q$ and can affect everything from a cell's life cycle to electrical signaling and muscle contractions. In this study, mechanical stretch increased the cells' calcium signaling responses when placed under hyperosmotic stress by the addition of salt. This increase was much larger in the cocultured cells and in isolated PC12 cells as compared to isolated WKO cells. Additionally, when the two cell types were cocultured and neuromuscular junctions formed, there was a noticeable increase in neurite formation and branching in the cells, and the two cell types converged upon a similar calcium response. Furthermore, a small population of the cocultured cells did not respond to the salt stimulation with either a calcium response or the morphological "shriveling" characteristic of the hyperosmotic stress. This suggests that the formation of the neuromuscular junctions may enhance resistance to stress and deformation in the two cell types. Understanding the role of these neuromuscular junctions in maintaining cellular health and disease prevention is crucial for growing mature muscle tissue in vitro, advancing our knowledge of how diseases caused by impaired NMJs function, such as Myasthenia Gravis and Lambert-Eaton Myasthenic Syndrome, and opening the door to future research into the various ways NMJs enhance the health of their component cells.

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Acknowledgements

I would like to begin by acknowledging Worcester Polytechnic Institute for providing me with the opportunity to gain hands on experience in Professor Scarlata's lab and to work on this project. WPI has given me the critical thinking and teamwork skills that I will continue to employ on my path to becoming a veterinarian. This project would also not be possible without the support of my advisor, Dr. Suzanne Scarlata. Her support, feedback, and amazing enthusiasm for the field shaped this paper and my lab experience into what it is today. Furthermore, I would like to thank Androniqi Qifti for welcoming me into lab and setting me on the path to success with her guidance, patience, and willingness to teach me all the of the lab techniques I would need and more. My research would have been slow-going without her support. Additionally, I extend my gratitude to Prof. Kristen Billiar and his graduate student Zachary Goldblatt for allowing me to use their stretch device throughout the course of my project, and for providing feedback that led to one of the key breakthroughs of my project. Finally, I would like to thank my family and friends for sticking by me these past four years and especially for supporting me through the pandemic and my senior year. I would not have been as successful without their encouragement.

Rationale

The purpose of this project was to better understand how differentiated smooth muscle and neuronal cells vary their calcium signaling response with regards to repeated physical stress, and how the formation of neuromuscular junctions between the two cell types affects this response. Calcium levels play an important role in many processes across the cell, from affecting the cell's lifecycle to electrical signaling, and many enzymes and proteins are sensitive to these levels. Additionally, although their importance to normal muscle growth and disease prevention is well known, the exact role and function of neuromuscular junctions in vivo is largely a mystery. Therefore, the repeated stretching of the cells in this study mimics the continuous oscillating motions of muscles and their attached neurons in the body, providing a more-accurate system for studying this vital cellular response.

Background

(Need to make diagram of GPCR pathway and neuromuscular junction)

GPCR Pathway

G-protein coupled receptors (GPCRs) are one of the largest families of mammalian receptors found in the cell membranes of eukaryotic cells that play a vital role in a multitude of cellular functions, including perception of sensory information, hormone release, regulation of cell contraction, and cell growth or differentiation[1]. Because of their importance in intracellular signaling for such a vast array of different processes, GPCRs serve as a valuable target for drugs. Approximately one third of drugs currently on the market target GPCRs, and there are far more published leads for future drugs targeting the GPCR gene family than there are for any other families like Cystine Proteases or Ion Channels[2,3]. Most importantly for this experiment, however, is the ability of GPCRs to regulate the release of intracellular Ca^{2+}. This occurs through the $G\alpha_q$ pathway, one of the four pathways coupled to GPCRs, which is displayed in Figure 1. In the $G\alpha_q$ pathway, the GPCR interacts with the three heterotrimeric G-proteins Gα, Gβ, and Gγ.

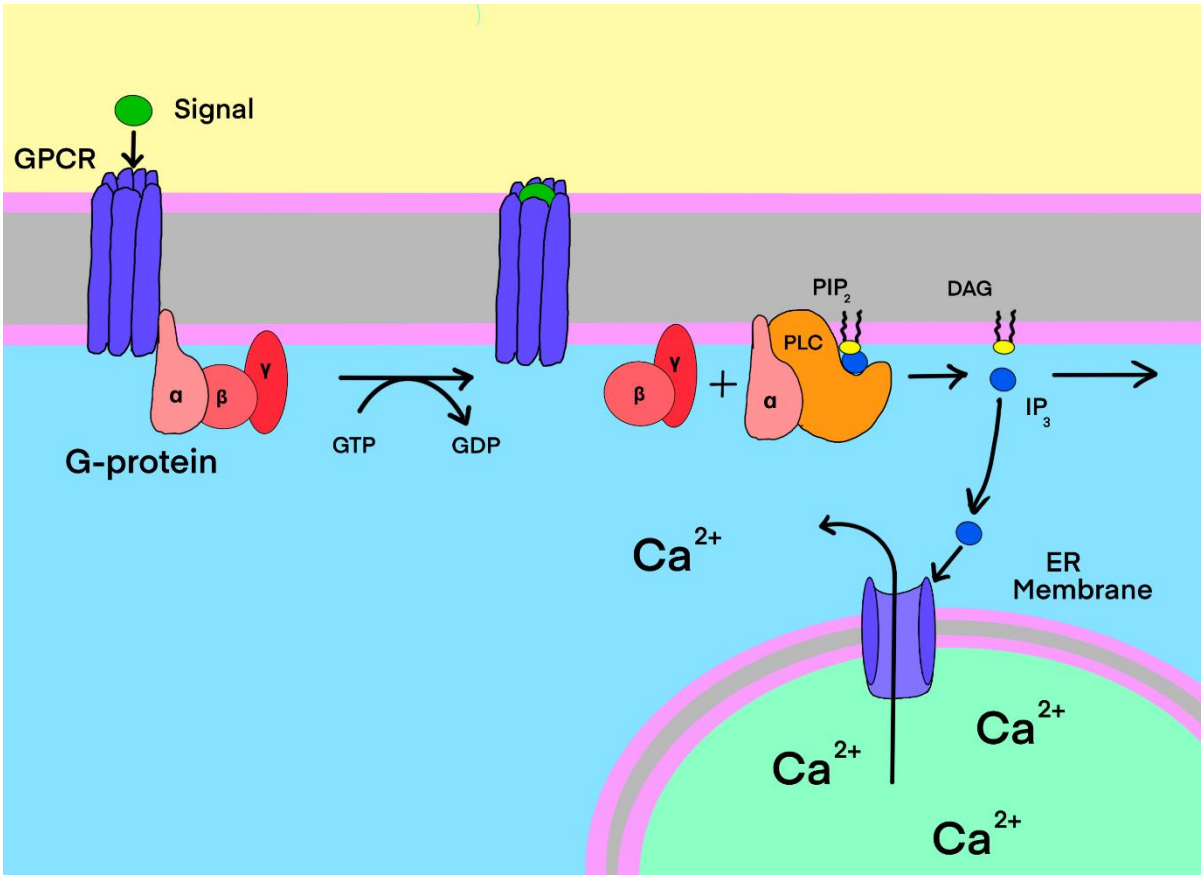

Figure 1: *A diagram of the $G\alpha_q$ pathway. An extracellular signal molecule binds to the transmembrane GPCR, exchanging a GDP for a GTP in the Gα subunit, causing it to dissociate from the remaining subunits and bind instead with PLCβ. This catalyzes the cleavage of PIP_2 into DAG and IP_3. IP_3 then binds to channels in the ER membrane, leading to the release of Ca^{2+} into the cytosol.*

When the receptor binds a ligand (commonly dopamine, acetylcholine, carbachol, or certain hormones, among others), it catalyzes the exchange of a GDP for GTP in the Gα subunit, and the subunit loses its affinity for the $G_{\beta\gamma}$ complex. Instead, $G_{\alpha q}$ associates with phospholipase Cβ (PLCβ). Now activated, PLCβ can catalyze the hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP_2) into inositol (1,4,5)-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is released into the cell where it diffuses and eventually binds to channels on the Endoplasmic Reticulum, promoting the release of Ca^{2+} into the cytosol.
The elevated levels of Ca^{2+} has the potential to lead to many cellular responses. For instance, it can cause Protein Kinase C to migrate to cell membrane where it is activated by DAG, leading to a host of proliferative and mitogenic changes in the cell[4,5]. Additionally, in smooth muscle cells the increased Ca^{2+} can stimulate contraction of the cell, or activate Ca^{2+}-sensitive transcription factors[6]. Known as "excitation-transcription coupling", this process translates short-term calcium signaling and contractions over time into long-term regulation of a smooth muscle cell's transcriptome[7]. Calcium signaling is equally as important in neuronal cells as well. Not only do Ca^{2+} levels control secretion of neurotransmitters from neurons, but they are also vital to the induction of long-term depression at certain synapses, which is believed to be a cellular mechanism responsible in part for cerebellar motor learning[8]. Furthermore, calcium in neurons helps control the process of memory formation and consolidation, the regulation of specific genes, and the depolarization of the plasma membrane[9].

Neuromuscular Junctions

Neuromuscular junctions (NMJs) are synaptic connections between the ends of nerve cells and muscle cells and serve as sites for the transmission of action potentials and signaling molecules between the two types of cells[10]. The NMJ can be broken down into three main areas: the nerve terminal (presynaptic membrane), the motor endplate (postsynaptic membrane), and the synaptic cleft (area between the two membranes), which are detailed in Figure 2. Over the course of neuromuscular transmission, electrical activity in the motor neuron triggers an influx of Ca^{2+} in the nerve terminal, prompting the release of synaptic vesicles containing certain chemical transmitters (often acetylcholine, one of the ligands that triggers the $G_{\alpha q}$ pathway) from the end of the nerve terminal into the synaptic cleft. After crossing the roughly 50 nm gap, the neurotransmitters contact the motor endplate. The motor endplate is a thickened area of the muscle's cell membrane that contains folds known as junctional folds. These folds contain receptor's that accept the neurotransmitters, opening ion channels that create a localized action potential in the motor endplate, serving as a starting point for muscle contraction[10]. Although this study focuses on smooth muscles cells, neuromuscular junctions are formed on cardiac and skeletal muscles as well, making them a critical feature for the lives of humans and animals alike. They are responsible for transforming electrical signals into muscle contraction, therein doing everything from allowing muscle movement to regulating one's heartrate. Therefore, when this intricate system of signal transmission is threatened or impeded in any way, it often leads to muscle weakness, paralysis, difficulty breathing and/or a severe degradation in quality of life[11]. Some well-known diseases related to faulty NMJs are Myasthenia Gravis, Lambert-Eaton

syndrome, and Botulism[10]. Treatments for diseases such as these are lacking, however, so gaining a better understanding of the structure and function of NMJs is essential to pave the way for future research and medicine. Furthermore, whether it's the rhythmic pumping of the heart, one's arteries, or exercise, muscle cells and the neuronal cells they've formed connections to are constantly stretched in rhythmic motions in vivo. Therefore, the repeated stretching of the cells in this study mimics the continuous oscillating motions of muscles and their attached neurons, providing a more-accurate system for studying this vital cellular response.

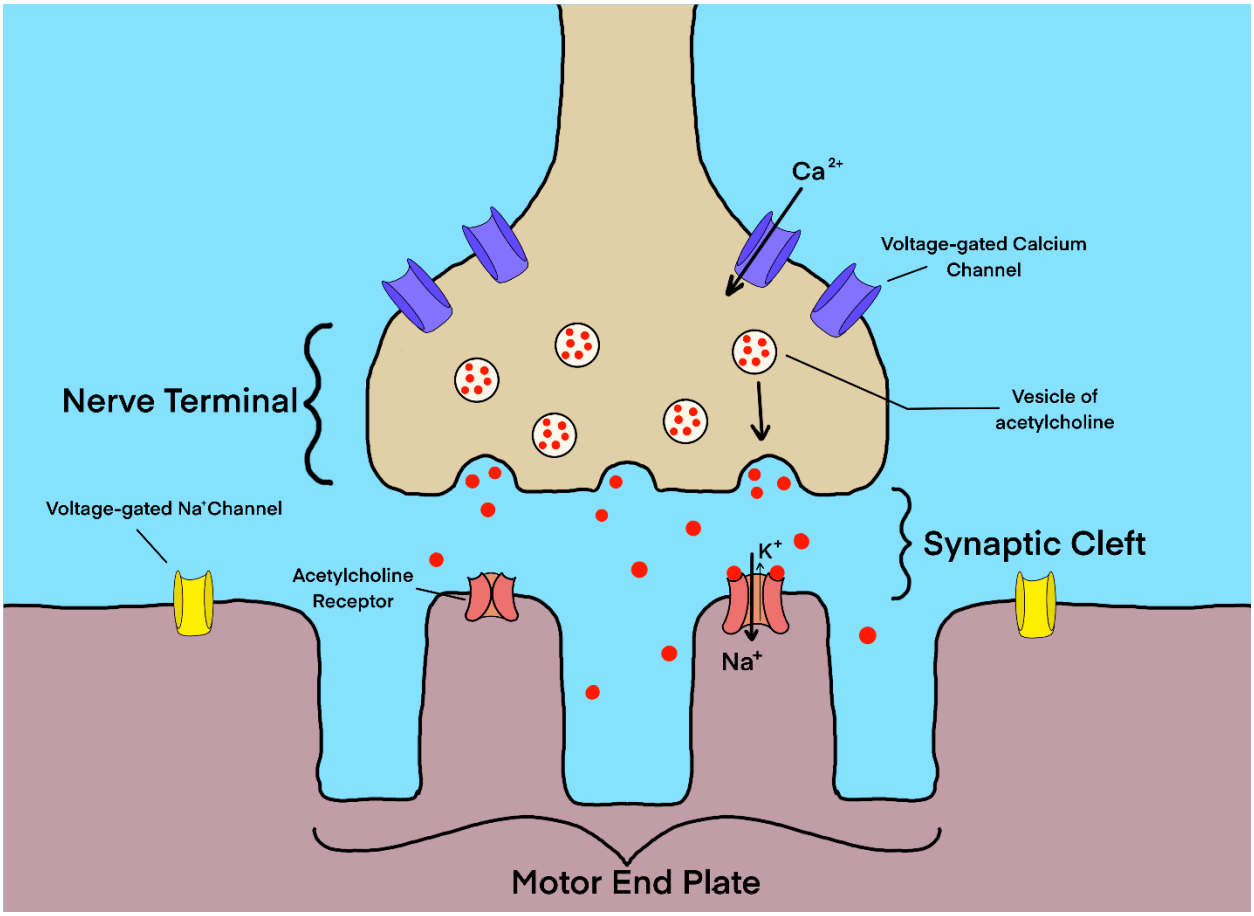

Figure 2: *Diagram of a neuromuscular junction. The NMJ is divided into three main areas: the nerve terminal, the synaptic cleft, and the motor end plate. In the nerve terminal, the activation of voltage-gated calcium channels and the subsequent influx of Ca^{2+} causes vesicles of the neurotransmitter acetylcholine to be released into the synaptic cleft. The acetylcholine is then received by receptors on the folds of the motor end plate, triggering an influx of Na^{+}, leading to a localized action potential.*

Caveolae

Caveolae are a common, but important, morphological feature of the plasma membrane among many types of mammalian cells, although they are notably absent from neuronal cells[12,13]. They present themselves as invaginated uncoated pits that are bulb-like in shape, and depending on the quantity of them in the membrane, they have the potential to increase the surface area of the cell

by nearly 100%. They have even been implicated in cardiac and muscle diseases such as muscular dystrophy[12]. These pits are roughly 50 nm across and 50-100 nm deep and take part in various biological processes such as endocytosis, transcytosis, and signal transduction[13]. Although their composition is high in sphingolipids and cholesterol, similar to lipid rafts, the presence of caveolae-specific proteins differentiates them from each other. These three proteins (caveolin-1, 2, and 3) form scaffolding domains that localize many signal transduction proteins to within the caveolae. For instance, the presence of caveolae (and the proteins within them) have been shown to directly affect Ca^{2+} signals mediated through the $G_{\alpha q}$ pathway. Caveolin-1 and Caveolin-3 specifically bind to the $G_{\alpha q}$ subunit, with an increased affinity after $G_{\alpha q}$ is activated, leading to an extension in the magnitude and duration of Ca^{2+} responses[14].

Methods

Cell type:

WKO-3M22 (or just WKO) cells are undifferentiated and rapidly proliferating smooth muscle cells derived from the Wystar Kyoto Rat. Although originally obtained from ATCC, the cells were gifted to the lab by the generous Dr. Marsha Rolle.

PC12 cells are undifferentiated neuronal cells derived from a rat pheochromocytoma (a tumor of the rat's adrenal gland). Obtained from ATCC, these cells differentiate relatively quickly in the presence of NGF and serve as a good model for studying neurons.

Cell culture:

The WKO cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% Fetal Bovine Serum, 1% P/S, 1% Sodium Pyruvate 1% L-Glutamine, and 1% Non-essential Amino Acids. When the cells were split, 2 mL of Trypsin were added, and the dish was gently rocked and tapped until the cells detached from the plate.

The PC12 cells were maintained in high-glucose DMEM supplemented with 10% Horse Serum, 5% Fetal Bovine Serum, and 1% P/S. All cells were incubated at 37°C in 5% CO_2.

Sample Preparation:

As a means to prepare the cells for bi-directional stretch, a specialized PDMS plate was created using the Sylgard 184 Silicon Elastomer Kit (Ellsworth Adhesives) and a special mold provided by Dr. Kristen Billiar's Lab. After curing, the PDMS plate required coating in order for the cells to adhere to their surface.

For experiments involving only WKO cells, the plates were sterilized and cleaned with 70% ethanol, then washed with Hank's Balanced Salt Solution (HBSS). After aspirating the HBSS, the surface was covered with a Fibronectin solution and allowed to incubate at 37°C before removing the Fibronectin, washing once more with HBSS, and returning the Fibronectin solution to the PDMS to incubate for another half hour. The double coating of Fibronectin was necessary for the WKO cells to properly adhere to the surface. The plate was then seeded with WKO cells, and the next day the media was switched to WKO Differentiation media, consisting of DMEM supplemented with 1% FBS, 1% L-Glutamine, 1% P/S, 1% Insulin-Transferrin-Selenium, and 50 µg/mL Ascorbic Acid. The cells were allowed to differentiate for 2 days before imaging.

For experiments involving PC12 cells (cultured alone or co-cultured with WKO), the fibronectin coating was insufficient for the PC12 cells to adhere. After curing, the PDMS was treated with Sulfo-SANPAH (Thermo Fisher). The Sulfo-SANPAH solution was diluted in 50 mM HEPES buffer at 10 µL solution per 1 mL HEPES buffer. The surface of the plate was then covered with this new solution and placed under UV light for 15 minutes before being aspirated and washed

with Phosphate Buffered Saline (PBS) 3 times over the course of 10 minutes. This Sulfa-SANPAH treatment serves as a heterobifunctional crosslinker to allow much better attachment of collagen to the PDMS surface in the following steps. The surface of the plate was then covered in Collagen Type I (Corning), which had been diluted to 0.02 Mg/mL in Acetic acid, and the plates were left overnight in the fridge. The following day, the collagen was aspirated, and the surface was washed with PBS. In order to sterilize the plate, the plate was filled with an antibiotic solution containing 100 µg/mL Pen-Strep (MediaTech), 2.5 µg/mL Amphotericin B (Corning), 10 µg/mL Ciprofloxacin (Alpha Aesar), and 100 µg/mL Gentamycin (Alpha Aesar) until the surface was covered. The plate was then sterilized overnight in the fridge.

For experiments involving only PC12 cells, to differentiate them, the plate was first seeded with PC12 cells, and the next day the media was changed to PC12 Differentiation media consisting of DMEM, 1% P/S, 1% Horse Serum, and NGF at a concentration of 1 µL/mL. The cells were allowed to differentiate for 2 days before imaging.

For experiments involving the coculturing of PC12 and WKO cells, to differentiate the cells together, the plate was first seeded simultaneously with PC12 and WKO cells at final concentrations of $5.00*10^4$ cells/mL and $5.25*10^3$ cells/mL, respectively. Then, the following day the media was changed to a differentiation media consisting of DMEM, 1% P/S, 2% Horse Serum, 2% FBS, and NGF at a concentration of 1.5 µL/mL. The cells were allowed to differentiate for 2 days before imaging.

Calcium Studies:

To prepare the attached and differentiated cells for imaging, the cells were labeled with a fluorescent calcium indicator (Calcium Green, Invitrogen) that allowed the cells' calcium levels to become visible through the microscope. To label the cells, the differentiation media was aspirated, and the cells were washed with HBSS. Then, a solution consisting of 4 µL of Calcium Green diluted in 2 mL of HBSS was added to the plate, and the plate was incubated at 37°C for 1 hour, after which the plate was washed with more HBSS to remove residual Calcium Green before imaging.

Stretch Experiment:

The device used to apply the bi-directional stress was borrowed from Dr. Billiar's lab and was specially fitted for the PDMS plates created with the mold from the same lab. Software named "Processing", written by Ben Fry and Casey Reas was used to operate the device, and allowed for the plates to be stretched in the x and y directions independently or simultaneously, and with specific magnitudes of stretch and stretch frequency. For the purpose of these experiments, the cells were stretched simultaneously in both directions once per second for varying amounts of time and stretch. The times tested were 5 and 10 minutes of stretch, at 2% and 5% stretch. Longer stretch times and greater stretch magnitude than these conditions have proven fatal to

cells in the past. After stretching, the cells were stimulated with a 5% KCl salt solution, and their Calcium responses were imaged.

To image these responses, a Zeiss LSM 510 confocal microscope with a 488 nm laser was used, along with a 10x objective lens. Any higher magnification objective lens was unable to focus on the cells through the PDMS. For each cell-type, including the cocultured plates, two non-stretched controls were imaged: one without salt stimulation, and one with salt stimulation. Using the Zeiss, a series of 400 images of the brightfield and Calcium Green channels for each condition were taken over the course of about 13 minutes. For cells imaged with salt stimulation, the plate initially contained 700 µL of HBSS, and after image ten, 300 µL of the salt solution was added to the plate, and the microscope was refocused.

Results

For this study, rat smooth muscle cells (WKO-3M22), and rat neuronal cells (PC12) were used to investigate the calcium response of these cells when stimulated with a KCl salt solution after varying magnitudes and durations of oscillating bi-directional stretch, as well as how the interactions between the two cell types when cocultured affected this response under the same experimental conditions. First, the cells were plated onto PDMS plates treated with the proper coating for their cell type(s), and the cells were differentiated. Then, the cells were treated with the fluorescent calcium sensor Calcium Green, and were stretched for 5 or 10 minutes at 2% or 5% stretch. After, the cells were stimulated with a KCl solution and imaged over a period of about 800 seconds. Each cell culture reacted to the sets of conditions in its own unique way. For instance, Figure 3 displays the control experiments for each cell type. All cells were exposed to the same two conditions: no stretch with salt stimulation, and no stretch with no salt stimulation. Despite this, their calcium responses (when stimulated) varied significantly.

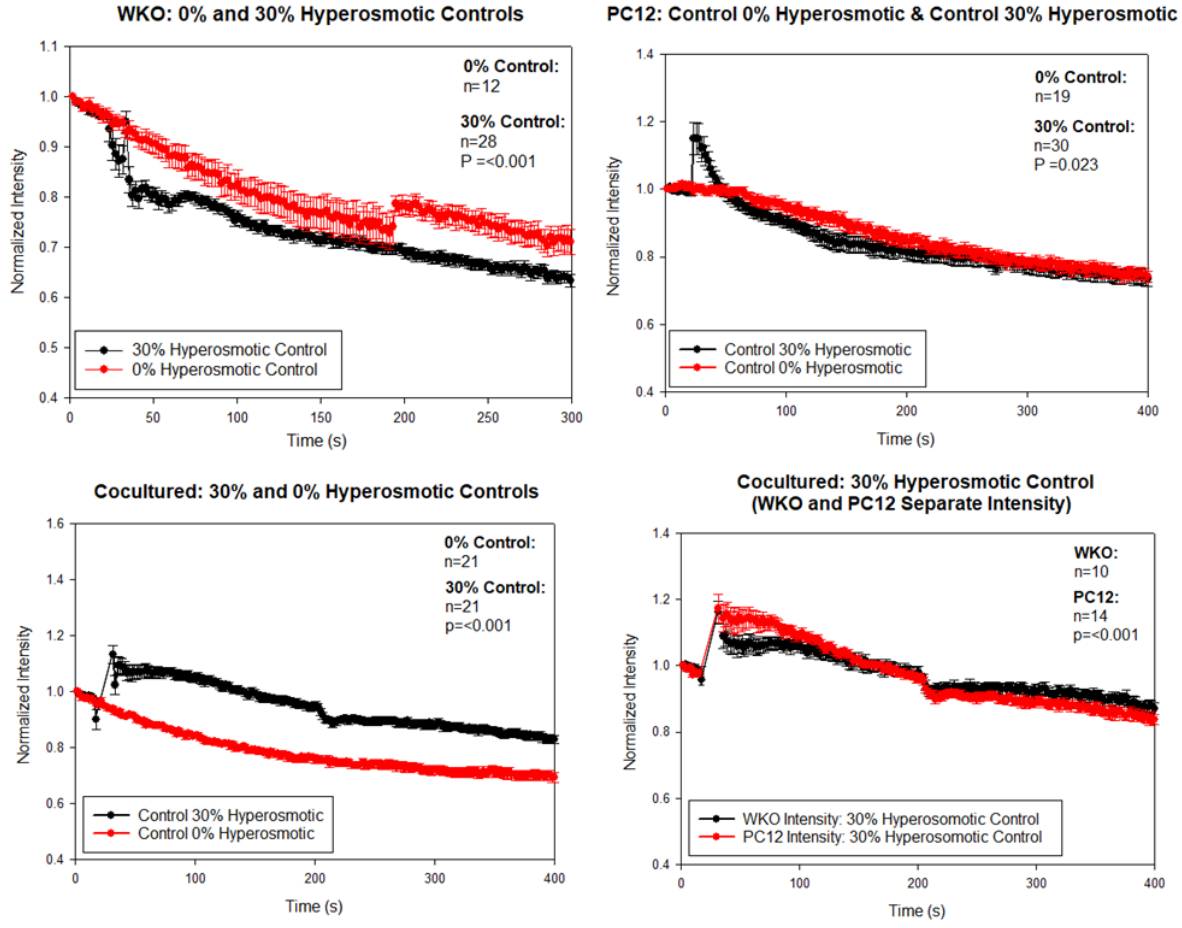

Figure 3: *Control experiments for all three cell cultures. The black line shows the normalized calcium intensity after stimulation with a 30% hyperosmotic solution, while the red line tracks the calcium intensity with no stretching or stimulation. Bottom right graph is the Cocultured 30% Control with the intensities of the WKO and PC12 cells plotted separately, showing their behavior is not simply due to the two cell types' intensities being averaged.*

After adding the salt solution to unstretched WKO cells, the calcium levels initially fall off sharply, but then spike upwards and dramatically drop before leveling off. On the other hand, in PC12 cells the calcium levels rise well above the initial amount after the addition of salt, and over time return to those shown by unstimulated cells. When the two cell types are cocultured, however, the calcium response to the salt solution is entirely unique, remaining well above the levels of the unstimulated cells for the duration of the experiment. This suggests that the interactions of these two cells are changing the calcium response and the $G_{\alpha q}$ pathway regardless of stretch.

In addition to the cell and culture type affecting the calcium response, the magnitude and duration of stretch also appears to interact with this pathway. As displayed in Figure 4, in WKO cells, the duration and magnitude of stretch did not noticeably change the initial increase in Ca^{2+} Green intensity, but it did affect the rate at which the cells recovered that calcium. Comparing the three different stretch conditions, the WKO cells that were stretched by greater amounts and for longer amounts of time recovered their intracellular calcium at an increased rate.

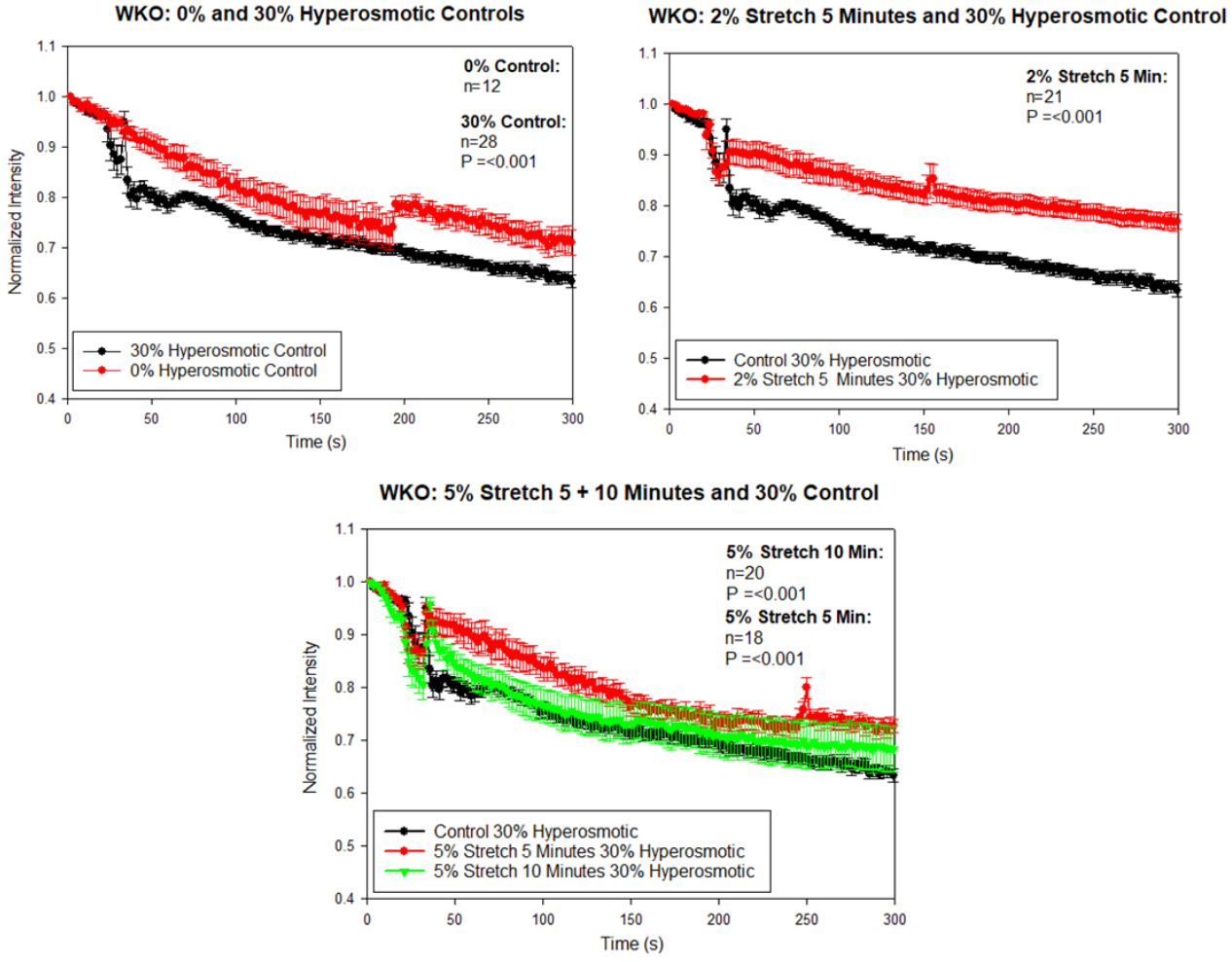

Figure 4: *Isolated WKO cells' calcium responses. Graphs of the intensity of the WKO calcium response for the control conditions (top left), at 2% stretch for 5 minutes (top right), and a combined graph of the 5% stretch condition for both 5 minutes and 10 minutes (bottom)*

This pattern did not hold true, however, for the PC12 cells. Despite lacking caveolae (which can extend the magnitude and duration of the Ca^{2+} response), PC12 cells exhibited a much stronger calcium response after stimulation with KCl, achieving normalized intensities far above their starting points, as Figure 5 shows. The calcium responses of the PC12 cells to different stretching conditions were distinct from one another but followed no apparent pattern. At a 2% stretch for 5 minutes, the addition of the hyperosmotic solution raised the Ca^{2+} Green levels of the PC12 cells by approximately 40% compared to their starting intensity. When the magnitude of stretch was increased to 5%, however, the calcium response very closely followed that of the hyperosmotic control, which was not stretched. Nonetheless, when the magnitude of stretch was kept at 5% and the duration was doubled to 10 minutes the calcium response dwarfed that of the previous conditions, raising the Ca^{2+} Green intensity of the cells by approximately 70% compared to their initial intensity, and never dropping below that initial intensity.

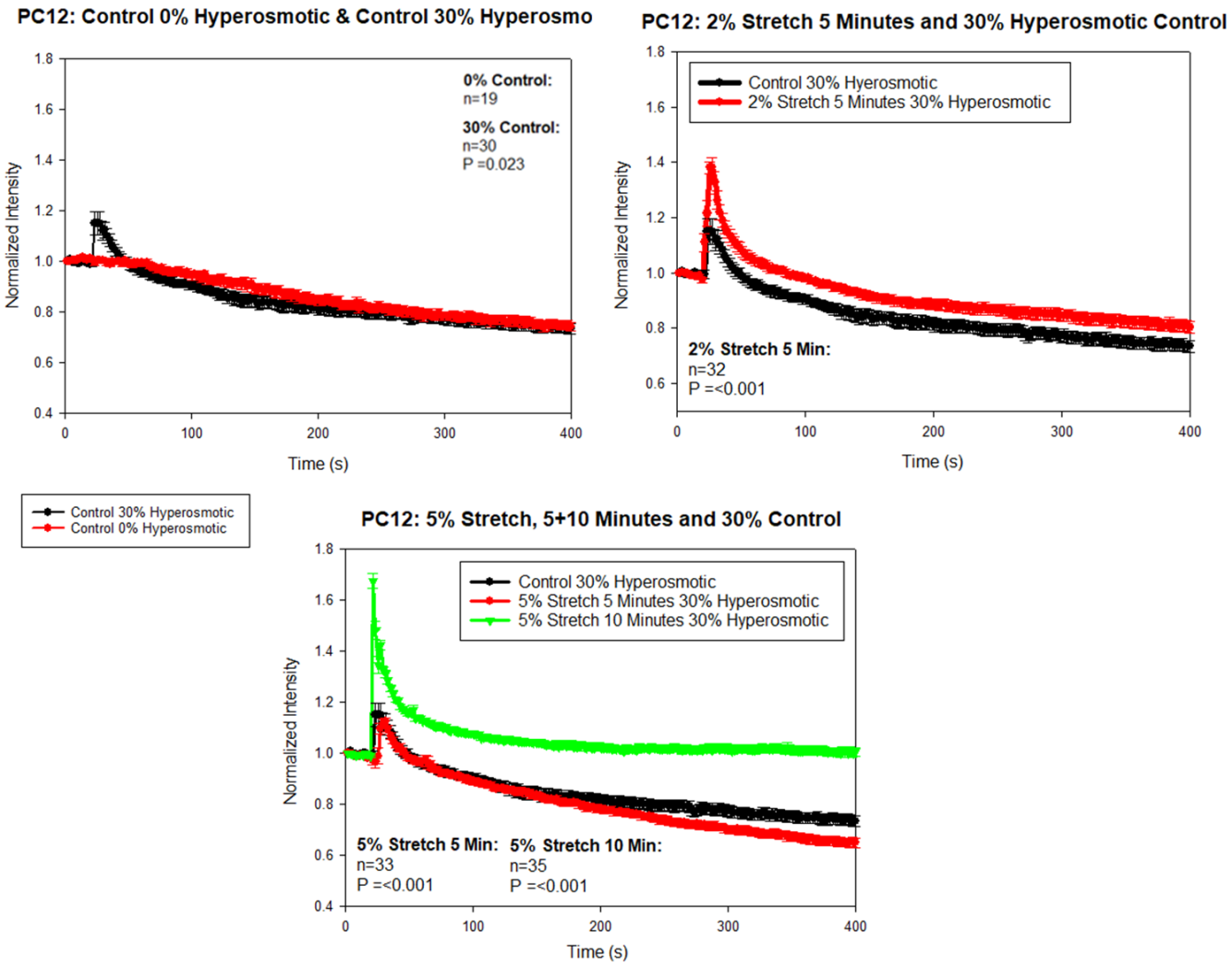

Figure 5: *Isolated PC12 cells' calcium responses. Graphs of the intensity of the PC12 calcium response for the control conditions (top left), at 2% stretch for 5 minutes (top right), and a combined graph of the 5% stretch condition for both 5 minutes and 10 minutes (bottom)*

The same experiments were then conducted on PC12 and WKO cells that had been cocultured and differentiated together, and once again the calcium response was unique when compared to the previous two cell cultures. Figure 6 demonstrates the manner in which stretch affected the calcium response after salt stimulation in these cocultured cells. These data were obtained by averaging the normalized intensities over time of both the neuronal and smooth muscle cells that were on the PDMS plate. These graphs show that stretching these cells increases the initial burst of Ca^{2+} Green intensity after stimulation when compared to the unstretched control, although no clear pattern exists with regards to whether magnitude or duration has a consistent effect on the intensity of this peak.

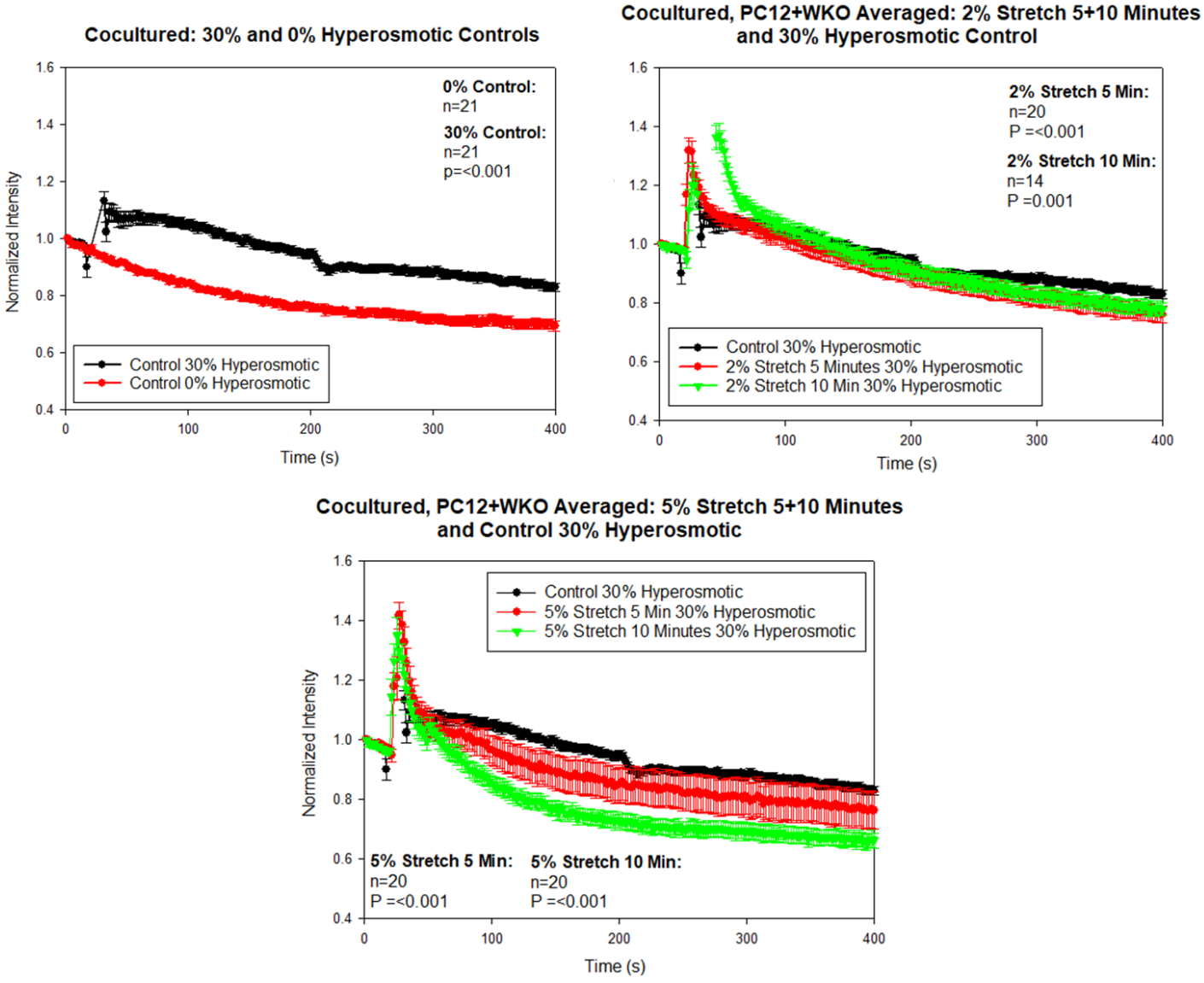

Figure 6: *Graphs of the averaged calcium responses for Cocultured cells (WKO+PC12). Averaged calcium response for the control conditions (top left), at 2% stretch for both 5 and 10 minutes (top right), and another combined graph at 5% stretch for both 5 minutes and 10 minutes (bottom).*

Furthermore, it is interesting to note that the calcium responses of these cocultured cells in Figure 4 have an elevated calcium peak consistent with the response in PC12 cells, and a calcium recovery pattern similar to the one observed in WKO cells, where a larger stretch for a longer duration increases the rate at which calcium is recovered. It may be tempting to assume this is because both types of cells have been averaged together, but the graph shown in Figure 7 demonstrates that this is not the case. Here, the WKO and PC12 cells on the cocultured plates have been analyzed and plotted separately. It is clear to see that when the two cell types are cultured together, their calcium responses are nearly identical. Both the WKO and the PC12 cells have a similar calcium intensity peak, and a similar calcium recovery pattern. It does appear, however, that the initial calcium recovery of the PC12 cells after the peak is slightly less than the WKO cells, but it does eventually overtake the WKO around the 130 second mark.

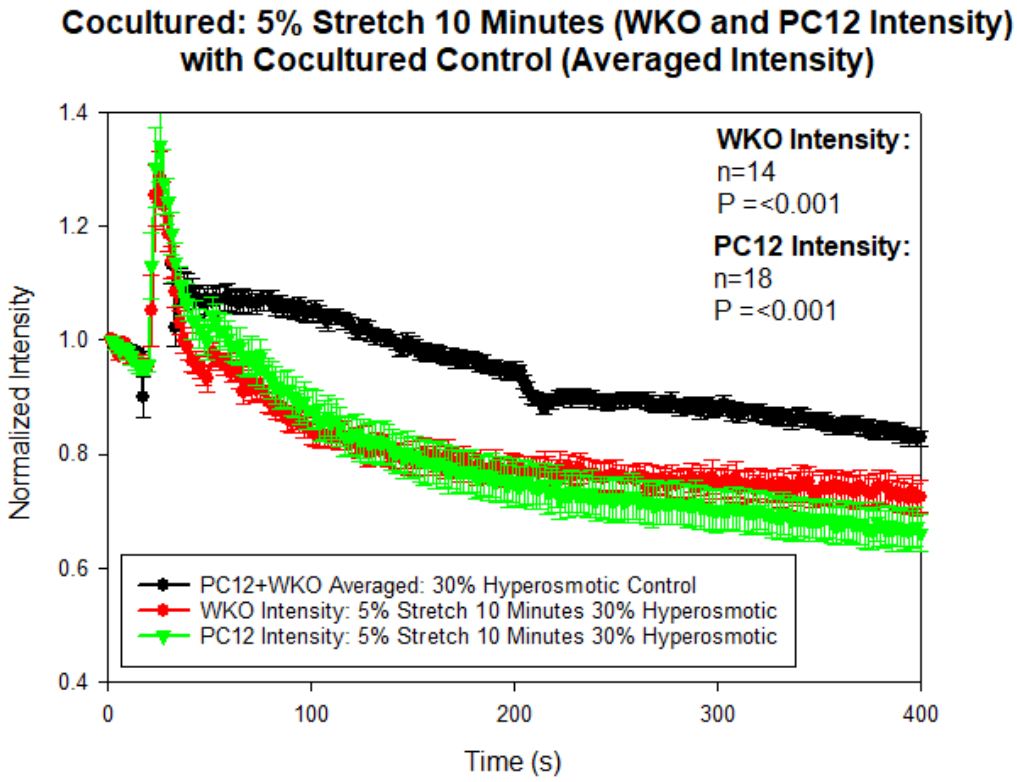

Figure 7: *Ca^{2+} Green intensity following stimulation with KCl of cocultured cells that underwent a 5% stretch for 10 minutes compared to the hyperosmotic control. The red line represents the plotted intensity of just the WKO cells from the cocultured dish, while the green line represents the plotted intensity of just the PC12 cells from the cocultured dish.*

There were also some important morphological changes that occurred in the cells when cocultured that were not seen when the two types of cells were cultured separately. These differences are highlighted in Figure 8. One of the most obvious differences is the increase in both length and number of neurites extending from the PC12 cells, connecting to other PC12 cells and forming connections with the WKO cells. Additionally, some of the WKO cells have taken on a new morphology when compared to images taken when they were cultured alone. They appear much flatter, more transparent, and cover a larger area on the PDMS plate.

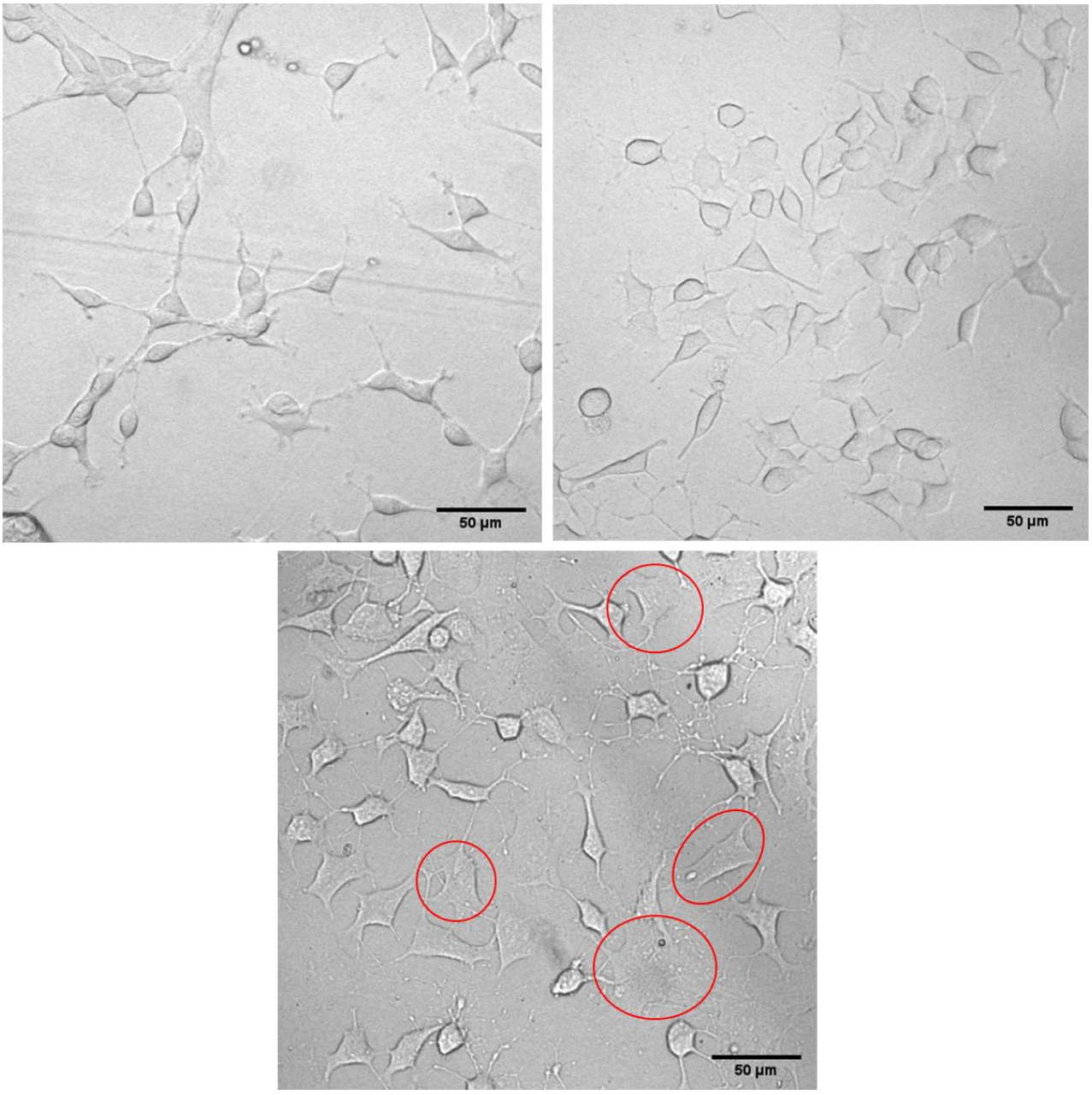

Figure 8: *Brightfield image of individually cultured WKO cells on PDMS (top left). Brightfield image of individually cultured PC12 cells on PDMS (top right). Brightfield image of cocultured cultured WKO and PC12 cells on PDMS. Red circles enclose examples of WKO cells displaying a novel morphology (bottom).*

It should also be noted that for every dish where a calcium response was recorded, the increase in intensity was accompanied by an apparent "shriveling" of the cells in response to the 30% hyperosmotic solution. This shriveling effect can be seen in Figure 9, which compares the Ca^{2+} Green and brightfield images for PC12 cells before and after the addition of the KCl. In that figure, the increase in Ca^{2+} Green intensity is immediately apparent, as is the sudden shriveling of the cells. There were two instances that occurred on only the cocultured dishes where addition of the hyperosmotic solution did not stimulate any calcium response. In those instances, the cells appeared entirely unaffected, as they also did not shrivel.

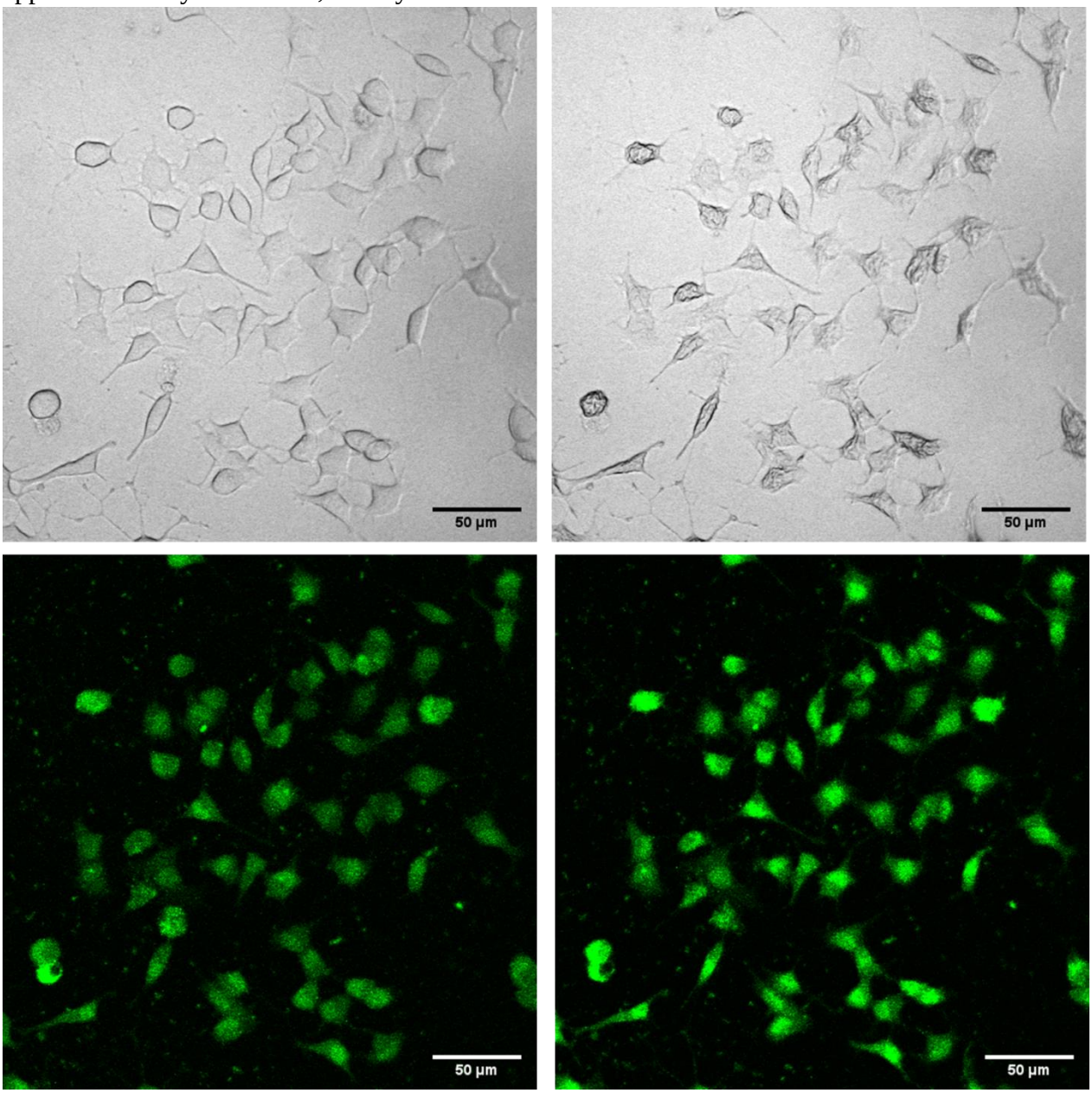

Figure 9: *Brightfield image of stretched PC12 cells before the addition of KCl (top left). Brightfield image of stretched PC12 cells immediately after the addition of KCl (top right). Ca^{2+} Green image of stretched PC12 cells before the addition of KCl (bottom left). Ca^{2+} Green image of stretched PC12 cells immediately after the addition of KCl (bottom right).*

Discussion

This study is the first to focus on the effect of physiological mechanical stretch on muscle and neuronal co-cultures, and the first to design a system for growing and differentiating neuronal cultures on a solid PDMS surface. We found that cocultures have different responses to water and ion flow, and strength, and will adapt distinct cell morphology when compared to the individually cultured cells. We initiated cell contraction by the addition of salt that would be comparable to the large ionic changes seen in the kidney. This replicates, for instance, conditions that the smooth muscle lining the ureter would experience.

In naïve WKO cells, no large effect on calcium was seen with the addition of salt. Differences in stretch, however, did affect the rate at which the WKO cells recovered their intracellular calcium. It is therefore possible that the stretching of the cells, in addition to affecting calcium release, may impact the SERCA pumps of the endoplasmic reticulum, a set a Ca^{2+} ATPases that transfer cytosolic Ca^{2+} into the ER lumen[15]. Different types of cells express different isoforms of these proteins, which may account in part for the variation in how stretching impacts calcium recovery in the ER when comparing the results of different cell cultures[15].

Unlike WKO cells, which were not greatly affected by the addition of salt, PC12 cells showed a transient increase and recovery of calcium. This behavior could be due to release and recovery of calcium from the ER to relieve the osmotic stress brought about by water efflux. This water efflux was the cause of the cells shriveling and may have impacted the calcium signal by increasing its effective cell concentration causing an increased signal. This is further supported by the few instances where cocultured cells did not react to the addition of salt by shriveling. In these cases, coupled with this lack of morphological change was a complete lack of calcium response. In addition to this water efflux, the applied stretch to the cells would be expected to alter the activities of ion channels and water channels in the cell membranes. The existence of mechanosensitive channels (MSCs), whose function is tied to mechanical stress, and the fact that mechanical stress can impact the kinetics of voltage-sensitive channels (like those that help regulate calcium signaling) by orders of magnitude are well proven[16]. Therefore, it is likely that the mechanical stretching was responsible for opening more ion channels and aquaporins in the cell membrane and increasing the calcium response in the PC12 and cocultured cells compared to unstretched cells. This seems especially likely after reviewing a study linking a lack of aquaporins in the brain tissue of rats to reduced calcium signaling, implying that overactive water channels in neuronal cells may contribute to an increased signaling response[17].

Most interestingly, however, is the change in morphology showing the impact that neuronal cells have on muscles cells through direct contact or through small vesicles excreted (i.e. exosomes). It is also clear that this contact with muscle cells distinctly affects the generation of neurites from neuronal cells. As previously mentioned, there were also two instances where the cocultured cells did not react to the salt solution through either a calcium response or morphological changes, suggesting that the interactions between the two cell types may help stabilize and support the cells. The relationship between this unique response and cell density

should be investigated in a future study. Additionally, at the chemical level, the contact between them appears to influence both cell types' calcium responses to the KCl solution. Despite having distinctly unique calcium responses when cultured apart, when cocultured, the WKO and PC12 cells converge upon a similar calcium response that appears to combine the elevated calcium peak consistent with the response in PC12 cells with the calcium recovery pattern observed in WKO cells. These findings have large implications in the field of disease research and for growing muscle tissues in vitro. Understanding how cocultured muscle and neuronal cells can alter each other's signaling responses and morphology, and knowing that mechanical stretch helps mediate cell recovery will be crucial to developing future treatments and therapies for neuromuscular degenerative diseases, and for more properly modeling the human body in vitro. This study lays the path for further research into the types of communication and connections occurring between the cocultured neuronal and smooth muscle cells, and how mechanical stress plays a role in these connections and in cell processes regulated by Ca^{2+} signaling.

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