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ATP-induced changes in F-actin expression Via $P2X_7$

Taylor Hircock
University of Northern Iowa

ATP-INDUCED CHANGES IN F-ACTIN EXPRESSION VIA P2X7 RECEPTOR ACTIVATION

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Taylor Hircock

University of Northern Iowa

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Abstract

The purpose of this research project was to study the effects that extracellular ATP has on F-actin levels in cells containing the P2X7 purinergic receptor. MAC-T cells which express the receptor were exposed to various levels of ATP and stained to undergo flow cytometry. In this case, F-actin was labeled with 488 phalloidin which was able to be detected by the flow cytometer. This was done in conjuction with controls to show how ATP exposure affected F-actin levels. Based on these results, the cytoskeletal changes taking place in the cell in response to ATP were able to be measured and studied.

Results showed that once ATP was present at high enough concentrations to sufficiently activate P2X7, F-actin levels increased. This increase was most intense shortly after treatment, peaking between 15 and 60 minutes post-exposure. The effects diminished after that point and by 2 hours post-exposure, F-actin levels began to decline. This data has possible implications in cell permeability during inflammatory processes and could serve as a basis for understanding the mechanism by which permeability increases.

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INTRODUCTION

The purpose of this research project was to study the effects that extracellular ATP has on actin levels and arrangement in cells containing the P2X7 purinergic receptor. It will first be necessary to provide a background on each of these structures as well as explain their relevance and impact to this project and to the larger body of scientific data.

Interest in this subject stemmed from related research on Bovine Respiratory Disease Complex (BRDC). BRDC comprises a number of illnesses commonly affecting cattle by infecting the respiratory tract. These are caused by a number of different pathogens, both viral and bacterial. The infections are typically exacerbated or brought on by certain environmental factors such as crowding and inadequate ventilation. There are several major clinical categories of BRDC, but shipping fever is the most serious and prevalent of these, so it remains the major focus. Shipping fever is an acute illness of adult cattle typically presenting with symptoms such as respiratory difficulty, mucous discharge, fever, depression, and respiratory tract lesions. When cows are transported or otherwise kept in tight quarters with poor ventilation or without regular feeding and watering, their natural defences are lowered and chances of respiratory infection increase. Inflammatory response is part of the disease process, which is where ATP signaling and the P2X7 receptor become especially important.

Adenosine 5'-triphosphate (ATP) is a modified nucleotide most well-known for its function as an energy transfer molecule during metabolism. However, ATP is also a signaling molecule in both extracellular and intracellular environments. More attention is being garnered currently regarding ATP's role extracellularly. Increasing evidence has shown that it plays an important role in many inflammatory processes.

Inflammation is a reaction produced in the body in response to a variety of stimuli such as infection, physical trauma, etc. Cells present in the tissue undergoing these stimuli respond by releasing chemical signals such as cytokines which prompt further cell migration and vascular changes. It is a corrective mechanism meant to protect tissues and stimulate repair (Lister et al 2007). There are many cellular and chemical components involved in inflammation and ATP can play a role in this process. The ATP-linked inflammatory process begins when ATP is present outside the cell. This is typically a "danger" signal to cells and may trigger inflammatory response mechanisms such as release of cytokines and increases in permeability (Lister et al. 2007). It then requires receptors to recognize it and begin sending additional pro-inflammatory mediators.

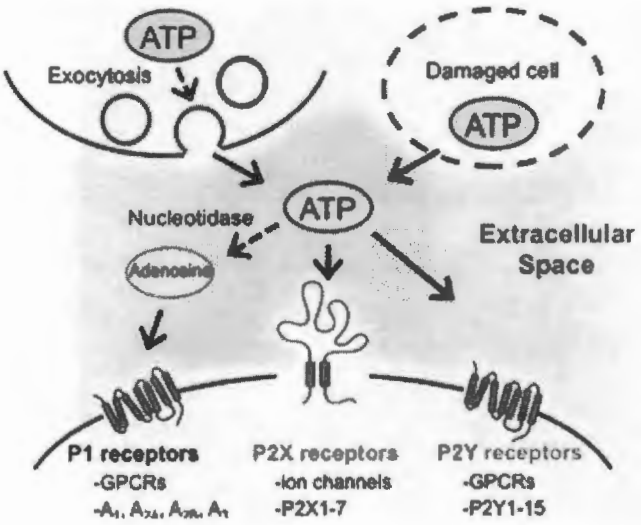

Figure 1. ATP Release and Extracellular Binding (Kawate 2012). This illustrates the possible origin points of ATP from live, dead, or damaged cells and the possible receptors it may bind to cause action

Receptors which bind to nucleotides, including ATP, are known as purinergic receptors, and contain several subgroups: P1, P2Y, and P2X. P2X receptors respond to ATP binding by forming ion channels. There are 7 subtypes within the P2X family, each having variations in structure, distribution, and activation time (North 2002). They are all common in mammals, with specific subtypes prevalent in areas such as the autonomic and sensory nervous systems, smooth muscle, cardiac muscle, and immune cells. P2X7 falls into the latter category, being expressed

chiefly on macrophages, lymphocytes, and microglia cells. It is also present in a number of epithelial cells, of which a specific line was used for this study.

The cells examined were bovine Mammary Alveolar Cells (MAC-T), an epithelial tissue which readily expresses the $P2X_7$ receptor. These cells are ideal not only for their receptor expression but also for their intrinsic qualities which allow for ease of use in culturing and experimentation. They have a doubling time of approximately 17 hours and can be cultured for hundreds of passages without showing diminished ability for growth, reproduction, or other properties (Huynh et al 1991). This allows numerous splitting of cell lines to encourage growth and produce cells for experiments. MAC-T cells exhibit a classic epithelial cobblestone pattern when attached and grown on a plastic surface, as shown in Figure 2.

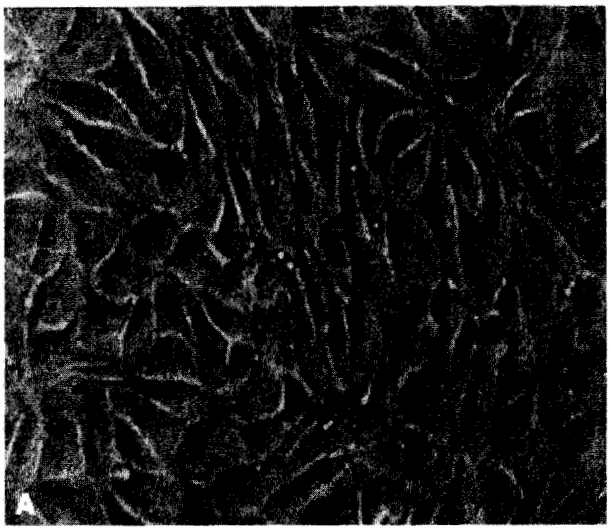

Figure 2. Cobblestone Cell Morphology (Leonard et al 1999). Photograph of tubular epithelium in cobblestone pattern, similar to MAC-T cells.

Flow cytometry is the use of lasers to detect fluorescently marked cells or molecules of interest. Cells are placed in solution and incubated with a fluorescent label for the desired marker, sometimes using antibodies which first attach to the marker and the fluorescent label. The solution is run through the machine and concentrated into a thin stream which allows cells to pass one at a time. Lasers are directed at this beam and the scattering produced by interference from cells allows the volume and density to be measured by forward scatter or side scatter (FSC and SSC respectively) sensors. When the beam hits fluorescent markers, they are excited and emit light which is also detected. These events together show the number, size, and density of

cells in a sample as well as amount of the labeled molecule. In this case, F-actin was labeled with 488 phalloidin which was able to be detected by the flow cytometer

Hypothesis

Through this research, I hoped to discover more about the cytoskeletal rearrangement taking place upon ATP exposure to P2X7 receptors, and its possibly link to cell permeability. The following questions were addressed through the experiments and data obtained:

1. How does ATP exposure affect F-actin levels in bovine epithelial cells expressing $P2X_7$?
2. What effect does length of exposure have on F-actin?
3. What effect do different ATP concentrations have on the extent of these changes?

My hypothesis prior to research was that F-actin levels would increase when the cells are exposed to ATP. This increase was suspected to begin at the minimum threshold (around 3 mM) and have a linear effect as ATP concentration increases, until a peak point when effects diminish.

Significance of Research

A few studies have visualized the cytoskeleton with confocal microscopy, but these did not quantify the amount of actin present in the cells. Additionally, no studies of this type appear to have been done using bovine cells of any type. This research provides solid evidence that F-actin levels increase with P2X7 activation. Armed with this knowledge, further inquiry may be made into how exactly the F-actin spike affects cell morphology and any effects that may have. It moves us one step closer to understanding the mechanism behind this pathway which has been shown to cause inflammation (Lister et al. 2007). There are possible clinical implications for the immune, respiratory, and vascular systems as well as others.

REVIEW OF LITERATURE

P2X7 Receptor

The P2X7 receptor was initially cloned from cDNA in 1997, and since that point it has been clear that it is predominantly expressed on cells of haemopoietic origin such as monocytes, macrophages, and microglia. As a result, activation of these cells results in increased production of pro-inflammatory chemicals including IL-1β, IL-18, IL-6, IL-8, and TNF-α (Lister et al 2007). In addition, prolonged stimulation eventually leads to membrane blebbing and cell death which releases more ATP into the extracellular environment, activating other cells. This serves to perpetuate the propagation phase of inflammation.

P1 and P2Y classes are G-protein coupled receptors, so their mechanism of action differs from that of P2X receptors which allow calcium and other cations into and out of the cell. This causes an equalization of charge around the cell membrane and depolarization triggers numerous processes within the cell to occur (North 2002). Compared to other P2X receptors, P2X7 is of average length (595 aa), but structurally unique. Its subunits only associate with itself to make homomeric pairs, whereas the other P2X subfamily members associate with each other to form hetero-oligomers (Ferrari et al 2006). It also expresses more forms than its relatives, more than 260 of which have been identified, although only a few of those remain functional. This is just the beginning of P2X7's distinctive traits.

The receptor has an extracellular domain, two transmembrane domains, and intracellular amino and carboxyl termini. The extracellular domain contains a binding pocket with lysine residues for the binding of ATP. The transmembrane domains are involved in pore formation and are flanked on the sides by pannexin channels and permeation pores which also open upon

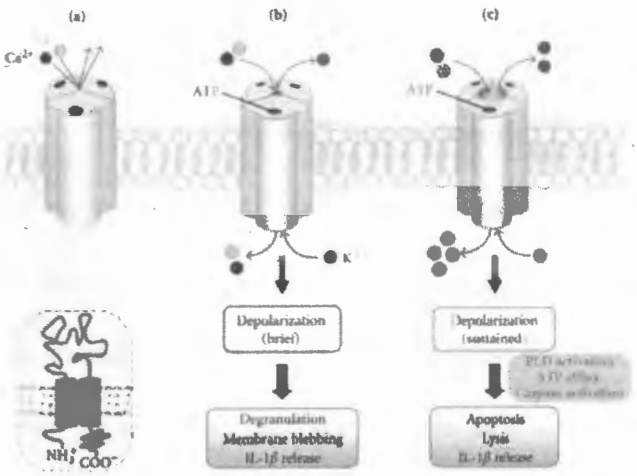

Figure 3. P2X7 Receptor Structure and Activation Effects (Skaper et al 2009). This figure shows the two main domains of P2X7 and effects of increasing stimulation on action

activation. The carboxyl terminus is important in sending and receiving signals within the cell (Wiley et al 2002).

P2X7 also differs in activation criteria. It is not selective only for ATP, but responds to other agonists and can be blocked by another set of antagonists. The ATP analog, bzATP, is the most notable agonist, being 10-30 times more potent in activating P2X7 (North 2002). Important to note though is that ATP remains the only major agonist present in physiological conditions. The effect of ATP, bzATP, and other agonists are greatly increased by reducing the concentration of extracellular ions such as magnesium and calcium (North 2002). These ions possibly serve as an allosteric inhibitor and are just one class of antagonists. Possibly the most crucial distinguishing trait is that P2X7 has a much higher activation threshold for ATP concentration (~3mM vs. 1-10μM) (Lister et al. 2007). P2X7 is a reversibly-binding receptor, so a pore forms in the membrane when it is activated by ATP, but will close once ATP is removed. After prolonged exposure, however, (approximately 15-30 minutes) the pore may close but the cell will remain irreversibly committed to death.

P2X7 causes more extreme changes resulting in DNA degradation and apoptosis, so extracellular conditions can be easily ascertained when these effects are taking place. P2X7 serves as an ideal marker for an ATP-regulated inflammation pathway, which contributes to further understanding of BRDC and other diseases, especially respiratory illnesses.

Activation Pathway

There are a few competing theories as to the actual mechanism of pore formation following activation. These theories are integral to the nature of this study and the results may shed light on them in one direction or another. The longstanding theory is that the P2X7 channel itself opens to form a pore depending on the amount and exposure time to ATP. More recently, however, a number of other theories have been proposed which suggest that P2X7 activation prompts coinciding processes with other membrane channels and transporters (Ferrari et al 2006). It should be noted that there are actually two separate membrane permeability events that take place: one small cation channel that opens within milliseconds of ATP binding, and a much larger second pore allowing molecules of up to 900 Da which opens after prolonged stimulation (Pelegrin 2011). The primary focus of this and other studies is the second and larger pore, as it has greater biological consequences.

The mechanism by which activation of P2X7 possibly stimulates actin formation is still under investigation. It is thought to be calcium-dependent, therefore happening secondary to calcium influx after the receptor is activated (Pubill et al. 2001). Some molecules that have been associated with the process are Rho-associated protein kinase (ROCK), p38 MAP kinase, and a number of phosphorylated lipids (Pfeiffer et al. 2004) (Kuehnel et al. 2009).

Previous studies have showed and measured permeability changes in P2X7-expressing cells once exposed to ATP. An increase in F-actin levels in my indicates a link between the two phenomena and suggest that they are related. Another suspected method of permeability increase is dissociation of intercellular junctions (Vandenbroucke et al. 2008). Though, it is possible that both of these contribute to overall permeability.

ATP Release and Role

ATP is surprisingly not alone in its ability to exert biological action on tissues and cells, a feature common amongst extracellular nucleotides and nucleosides (Pubill et al. 2001). In certain areas, especially the lungs, it does have the advantage of being easily available for release and in an area of high concentration for receptors specific to it. It has also been shown to be released with other neurotransmitters, possibly serving as an adjuvant to its effect. There are several significant findings which have stimulated interest in its role as a messenger rather than a metabolic source. One is that ATP binds to certain receptors (like P2X7) that no other nucleotides are able to (North 2002). Another finding is that it is normally present at very low concentrations (5-10 nM) in extracellular spaces, yet has potential for drastic upticks. This provides a wide range for regulation and reaching desired physiological effects.

The most common form of ATP-related inflammation takes place in conjunction with lipopolysaccharides (LPS). LPS is a type of molecule found on the membrane of Gram-negative bacteria which stimulate a heavy immune response. When LPS is detected by a cell, that cell begins producing IL-1β, a precursor to one of the central immune response chemicals, IL-1.

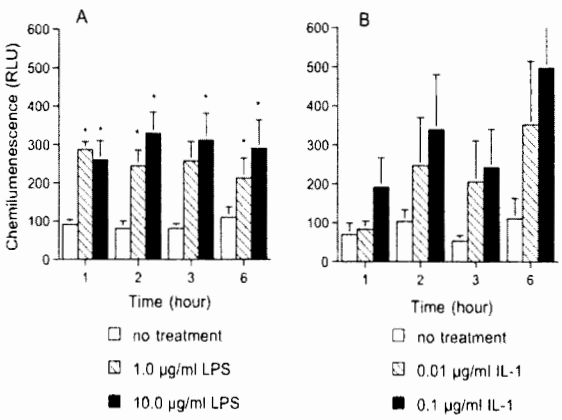

Figure 4. Causes of ATP Release (Craddick et al). These graphs demonstrate that LPS and IL-1 stimulate extracellular ATP release in lungs.

Although the cell is primed to produce IL-1, LPS stimulation does not complete this process and it requires a second signal. This signal has been conclusively proven to come by extracellular ATP. With this signal, IL-1β is released and converted to IL-1 with subsequent inflammation occurring (Ferrari 2006)

Studies have shown that hypoxia is one of the strongest signals for ATP release during acute inflammation. Other factors may include physical stress such as shearing, tonicity, and polymorphonuclear (PMN) cell activation. PMN activation was shown to increase extracellular ATP levels up to 6-fold, showing that it is an activation-dependent process (Eltzschig et al. 2006). In the lungs, experiments done by Craddick et al. (2009), endothelial cells did not release ATP in response to LPS, but epithelial cells and macrophages did. The origin of this ATP may be from dead host cells, dead bacteria cells, or exocytosis by live cells regulating inflammation. After release, much of this ATP is degraded or metabolized to ADP, adenine, or other molecules before it is able to reach a P2X receptor. Other forms may activate other inflammatory pathways via P2Y, P2Z, or other receptors.

Actin Arrangement

When ATP binds to the P2X7 receptor, pores form in the membrane. This allows ions, especially Ca^{2+}, to rush into the cell, serving either as a direct signal or secondary messenger causing rearrangement of the actin cytoskeleton (Pubill et al. 2001). This causes rapid morphological changes in the cell, taking place within minutes (Mackenzie at al. 2005). These disruptions in cell shape and structure caused by actin reorganization are suspected as a reason for the increased permeability between ATP-activated cells.

Some of the possible and likely actin arrangements in cells have been described in literature. In microglial cells, they shift from having a blunt protrusion on one end of the cell to having multiple highly branched processes, forming a root-like arrangement (Fang et al. 2009). Blebbing is the formation of lobe-like processes on cell perimeter, and is indicative of apoptosis, or programmed cell death. Blebbing has been observed in rats expressing human embryonic

kidney (HEK) cells (Mackenzie et al. 2005). Actin rings may also be present throughout the cell, at the leading edge, or elsewhere. F-actin is the filamentous form being described in these experiments and is what I will be measuring. It is comprised of many globular (G-actin) subunits which are polymerized into the F-actin form increasingly during inflammation. This is associated with the cell shape changes responsible for causing permeability changes (Langer and Chavakis 2009). Phalloidin fluorescent dye binds to F-actin but not G-actin and is therefore the ideal marker for use in this research.

MATERIALS AND METHODS

Culturing of Cells

MAC-T cells were initially removed from storage on liquid nitrogen, where they were dissolved in DMSO. Cells were partially thawed in a hot water bath at 37°C. Afterwards, 1 mL mixture of 50/50 sterile Dulbecco's Modified Eagle Medium (DMEM)/Fetal Bovine Serum (FBS) was added dropwise over 1-2 minutes to complete thawing. The solution was then placed in a 15 mL tube and DME/FBS mixture added until volume reached 10 mL. The tube was then centrifuged for 5 minutes at 3000 rpm after which a pellet was visible. The mixture was decanted and resuspended in 4 mL of culture medium and placed in a T-25 flask.

T-25 flasks containing cells were incubated at 37°C until confluent (typically 3-4 days). In order to sustain cell lines and encourage continued growth, DME media was changed intermittently by removing old media and adding 3-4 mL of fresh media. When necessary, cell lines were split into multiple T-25 flasks by removing media, washing twice with 5 mL of PBS, and adding 1 mL trypsin/EDTA to detach cells from the bottom of the flask. Media was added for volume and then centrifuged at 3000 rpm for 5 minutes. The cell pellet was decanted, resuspended in fresh media, and appropriated in 2-3 new flasks. The same process was used when preparing cells for experiment, but instead of placing resuspended cells in new culture flasks, they were evenly spread across a 6-well plate and grown to confluency.

Experiment Setup

Stock solutions were initially made for reagents needed. Dylight 488 Phalloidin was dissolved in 1 mL of pure methanol. ATP was massed and dissolved in ATP Free Water to a

concentration of 100 mM. 10 μL of Triton X-100 was diluted with 9.99 mL of PBS to create a .1% solution. Stock solution previously existed for 4% paraformaldehyde.

Once 6-well plates had a confluent layer of cells, they were removed from the incubator and treated with ATP. There were two wells each for control group, low ATP treatment, and high ATP treatment. For the first trial, The ATP was diluted in DMEM to concentrations of 1mM and 10mM. The contrast between these two concentrations was intended to give a clear picture of the effect that more ATP induces. The control wells were not treated with ATP at this step. They underwent the same treatment for the remainder of the experiment with the exception that in the final step of phalloidin addition, one control well did not receive this. This sample was used for flow setting optimization. Different treatment lengths were also used for each concentration of ATP to show the optimal time period for measuring ATP action. In subsequent experiments 10mM was used in all of the experimental wells at time periods of 15, 30, 60, and 120 minutes. Figure 5 below summarizes the 6-well plate setup for the experiments used in data collection.

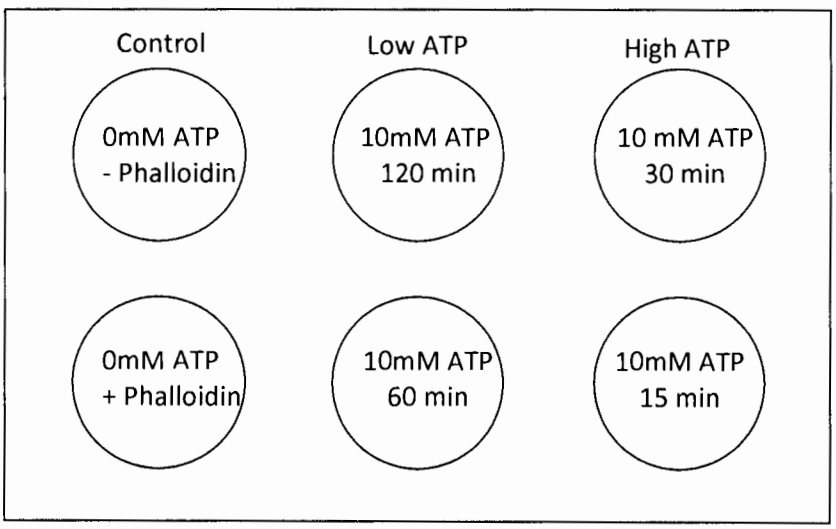

Figure 5. 6-well plate ATP Treatments. This figure displays the amount and length of ATP treatment prior to fixation and staining. Each well contains identical cells and experiment protocol beyond this step.

200 µL of 4% paraformaldehyde was added to each well and incubated at room temperature for 15 minutes. The paraformaldehyde solution was then removed and 250 µL of trypsin was added to each well to remove the cells from the plates. Cells were then placed in 5 mL round tubes and centrifuged at 3000 rpm for 5 min. When tubes were removed from the centrifuge, they could be held up to the light to show the presence of a faint but visible cell pellet. The tubes were decanted by pouring the liquid solution into a waste beaker while leaving the solid pellet attached to the bottom of the tube. The pellet was washed with 500 µL of 1X PBS two times, with the PBS being decanted each time. The PBS washes served to ensure that all trypsin was removed from solution so as not to affect future steps. 500 µL per tube of .1% Triton X-100 detergent was then added and mixed thoroughly to resuspend the cells and ensure exposure to the permeabilizing agent. The cells were incubated in this solution for 15 minutes at room temperature. This was meant to create pores in the cell membranes to allow phalloidin to enter the cells. 2 µL of phalloidin was added to each tube to create an approximately 1 unit/mL ratio, or 1:300. Once the phalloidin was added to each tube they were placed in the dark to be incubated for 30 minutes. After incubation, flow cytometry was performed on each tube to measure the amount of fluorescence which indicates phalloidin binding to F-actin.

Flow Cytometry

Samples were run on Applied Biosystems Attune Acoustic Focusing Cytometer. After priming the machine, the first sample used was a control sample with no ATP or phalloidin treatment. This was used to optimize settings for detection of cells within the proper ranges and adjustment of graphs to properly visualize results. PMT voltage was adjusted for forward and side scatter measurements showing the volume and densities of the cells to ignore small particles

thought to be only cell debris. It was also adjusted for the laser settings so that autofluorescence of unstained cells would not skew the fluorescence histogram. Instrument settings are shown below in Figure 6. Each sample was then analyzed and recorded until the total volume was used up, measurement time reached 5 minutes, or 10,000 events were recorded. The latter criteria is ideal because it gives a significant number to draw from when viewing plots and is a standard when performing statistical analysis.

Category	FSC	SSC	BL1
Voltage Threshold (mV)	1500	3000	1200

Figure 6. Flow Cytometry Settings. This table shows the voltage adjustment settings used for flow cytometry

Experiment Modifications

The first experiments done for this study were used to determine the proper time points and ATP concentrations needed to give us relevant results. Time points of 30 minutes and 1 hour were used along with ATP concentrations of 1 mM and 10 mM. The activation threshold of P2X7 lies around 3 mM and we decided to see if there would be a visible difference between concentrations above and below that range. Results from that experiment showed minor shifts in the fluorescence (indicating activation) at 1 mM, and more significant shifts at 10 mM. We postulated that some receptors were possibly being activated at 1 mM and not others, so to attain more conclusive results we chose 10 mM for future experiments. Both 30 and 60 minute periods gave interesting results, so we chose to use time periods on either side (15 min and 120 min).

Initially, our protocol followed the manufacturer's instructions included with the phalloidin. Unnecessary steps were removed in our protocol as phalloidin was binding directly to F-actin in the cells and no antibodies were needed. The suggested procedure was also designed

for 96-well plates rather than 6-well plates. Total volume for the plates is approximately equivalent, so addition of reagents was scaled based on mathematical equivalents for each well.

As with any scientific research, difficulties were encountered during and even before the experiment process. Situations were assessed and necessary changes were made to optimize the results of the study. Further complicating this process was that experimentation was started much later than planned due to things like funding and purchase of a new flow cytometer. Cell culture contamination proved only to be a problem once, but required reviving a frozen stock of MAC-T cells which set us further back. These circumstances forced us to quickly modify our procedure in order to attain useable data for analysis. During experiments, it was found that cell pellets didn't adhere as tightly to the 5 mL round bottom tubes, causing cells to be lost during washing and decanting steps no matter how gently done. This did not leave enough cells for sufficient analysis and prompted us to switch to 15 mL conical tubes which cells adhered to more tightly. Despite these difficulties, necessary changes were made and the process refined to give us not just good results but a better understanding of our materials and the scientific process as a whole.

Justification of Methods

The purpose of this experiment was seeking to discover how ATP exposure, dose, and time period to P2X7-expressing cells affected F-actin amount. Initial testing displayed that ATP dosing needed to be above the activation threshold of 3mM to give reliable results. Separate control samples and varying exposure length samples were used with identical cell populations to observe their difference in response. Incubation with phalloidin is specific to F-actin so flow cytometry is confirmed to be indicative of F-actin levels. Flow cytometry is the best method for providing quantitative measurement of these changes

RESULTS

Analysis of Flow Cytometry

A scatter plot of forward and side scatter measurements was made and a gate was created to include only cells in the main size population in daughter plots for further analysis. This eliminated outliers and small particles. The remaining cells were placed on a histogram depicting excitation wavelength and the number of cells at each wavelength. Due to the presence of only one cell population and treatment for each analysis, it typically resulted in a moderate to sharp peak which shifted depending on the time point. These shifts are the main indicator of F-actin levels and allowed trends to spotted and conclusions drawn from the data. Mean and median wavelengths were recorded, with median being the primary figure used to indicate fluorescence shifts. Figure 7 shows this data presentation format while comparing a no treatment control with phalloidin staining to a 15-minute exposure population.

An ANOVA test (Analysis Of Variability) was used to determine statistical significant variance in experiment results. Initial results did not fall within the .05 range of acceptable variation, but variation in fluorescence between experiments must be normalized, and plans are underway to consult a statistician to find a more suitable method of analysis.

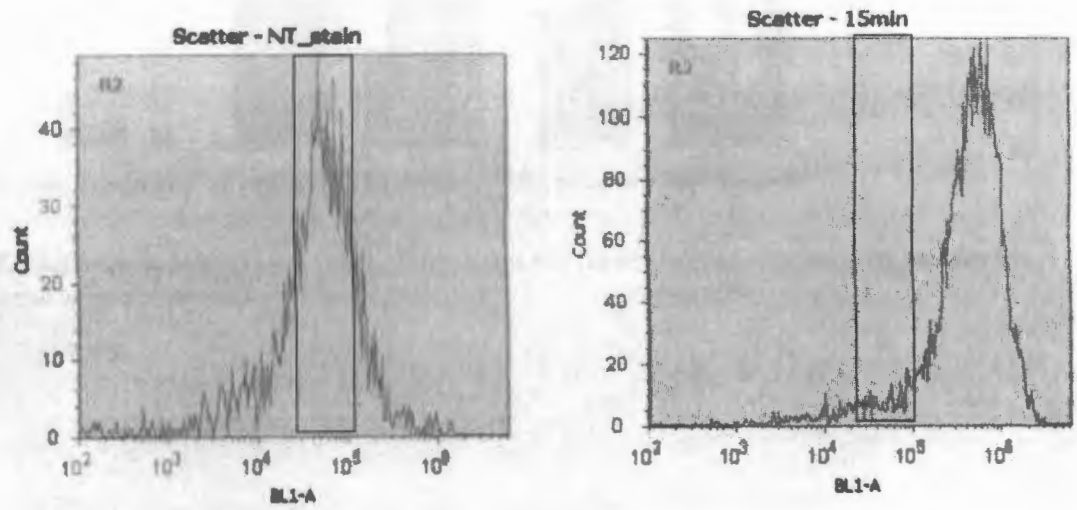

Figure 7. Fluorescence Curve Shift. On the left is the graph of the control treatment and right is 15 minute ATP treatment sample. Region markers are used to show the central area of the curve, which had a median shift to the right indicating greatly increased fluorescence.

Data

For the three experiments used in this study, median fluorescence data is the main focus because it quantifies the results to easily interpret, and it is more effective than mean at eliminating outliers. Fluorescence indicates the levels of F-actin present via binding and signaling by phalloidin. Increases in fluorescence show more F-actin formation, decreases show less formation or degradation. Actual quantities of F-actin cannot be ascertained by these tests, only relative values. The graph below summarizes that data and averages the median values to give comprehensive experimental data. Appendix A contains a table for the data shown below and data displays for every sample in one experiment representative of the others.

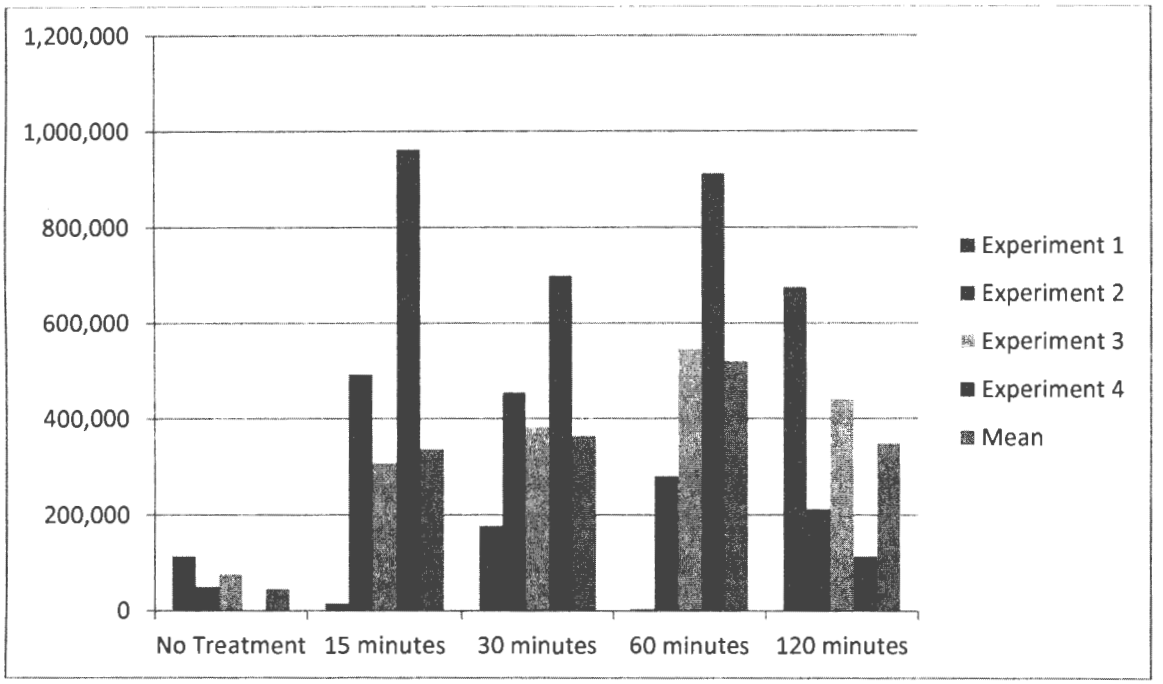

Figure 8. Fluorescence Median Data Graph. Summarizes and shows fluorescence results for controls and experimental groups of all trials.

As is evident from the above data, there is a drastic increase in fluorescence between the no treatment group and treatment groups for all experiments. This is most noticeable for the first 15-minute time period and slowly declines over the remaining time periods. Although experiment 3 continued to increase fluorescence up to the 60-minute mark, all of the experiments were in decline by 2 hours. Experimental variation is to be expected and may even display the wide range of time which the effects of P2X7 take to manifest within the cell's structure. The exception to this being Experiment 1 in which there was mishandling of ATP which allowed it to degrade prior to addition at 15 and 60 minute time points The uniformity of trends among the various experiments shows conclusive closure to the trend after 1-2 hours, eliminating the need to analyze further data points.

The rise in fluorescence after ATP exposure demonstrates a few things: ATP is present at significant enough concentration to activate P2X7, and activation is causing effects within the cell. The first of these is a positive confirmation of the experiment's intended effects. The second is a result of that yet a possible confounding factor. When P2X7 is activated for extended periods, apoptosis is a likely result. One concern was that this may lead to a decrease in cellular population or the presence of cellular debris that may skew fluorescence values. In actuality, cell numbers in the 60 and 120-minute samples were not decreased significantly enough to prevent the collection of a full 10,000 events. The second was prevented by increasing threshold levels for size and gating out dense areas low on the scatter plot which may not have been live cells. This gating can be viewed on the graphs in Appendix A. When gating was removed, there was a slight downward shift in the fluorescence curve, but not of great significance. It can be safely assumed that these issues were not considerable detriments to the quality of data obtained.

One curious issue was that some experiments had a biphasic curve at one time point. This was typically at a lower fluorescence and not as strong as the main peak, but shows that a certain cell population in the sample is responding different at that time point. In one experiment it occurred at 30 minutes and at 60 minutes in another. Because all samples received the same treatment at each step it is unlikely, but not impossible, that it results from a procedural error. No definitive conclusions have been reached about what might cause this biphasic response.

Non-Quantitative Observations

Certain phenomena were observed during experimental procedure that were unable to be captured or summarized by numerical data but are worth mentioning. The addition of ATP appears to have an effect on the binding of MAC-T cells to the plates. During initial experiments, 1mM treatments and controls took released from the plate less readily than 10mM treatments. In later experiments, treatment wells with longer exposure times also removed from the plate easier. Though it was no t visible through a light microscope, this may be due to morphological or structural changes of the cell. This may be linked to another strange observation, which was the decreased number of cells in the sample for control wells. After removal from the 6-well plate and centrifugation, pellets for control wells were consistently smaller and reached a lower event count during flow cytometry. When trypsin was unable to fully remove them from the plates a cell scraper was used so it would be assumed that the same amount of cells would be transferred at that point. The final note was that although .1% Triton X-100 detergent was used in most other studies, it may have been too strong of a concentration or agent for these cells. After permeabilization, cell pellets were noticeably smaller, possibly due to lysed cells. Less harsh reagents would have been used except for concern about possible effects on actin. These observations may or may not be significant, but are important to note for future work in this area.

DISCUSSION

Outcome of P2X7 Activation

As previously stated, the purpose of this study was to observe how F-actin levels changed in response to P2X7 activation. The data provides conclusive evidence that F-actin levels increase rapidly and decline after lengthy exposure. This switch from the uniformly-distributed G-actin which is normally present in cells to the filamentous form is a signal of cell or organelle motility, cell morphological changes, and associated results. F-actin formation and breakdown is a constant process which allows normal cell functions to occur, but changes of such a drastic and sudden magnitude as those measured indicate a direct response to conditions outside the cell.

F-actin's most thoroughly characterized role is its involvement in migration and motility. During this process, F-actin is formed at the leading edge of the plasma membrane into broken down into monomeric units at the tail end at a roughly equivalent rate (Saarikangas et al. 2010). This study demonstrates not just the formation of F-actin, but accumulation and retention for a specific purpose. It is assumed that some sort of well-regulated structure is being formed, although there is little consensus as to what that may be. F-actin is undoubtedly an indicator of a major intracellular response to an environmental signal regulated by ATP and P2X7.

Physiological Effects of Structural Changes

The changes taking place throughout this process are not restricted only to within the cell, but have even greater effects at the tissue and organ levels if activation is widespread. Studies show that increases in F-actin are correlated with increases in cell permeability to due structural shifts altering cell-to-cell junctions (Pubill et al. 2001). Looking specifically at vascular tissues and their role in respiratory exchange, these permeability changes are especially important.

Increased permeability in endothelial and epithelial tissues would allow blood products to leak out between cells where gap junctions and other structures would ordinarily serve as a seal. This fluid can severely hamper respiratory function by disrupting gas exchange at the alveoli. This is demonstrated by the figure at the right. In cattle, increased blood and fluid levels are commonly found in cows with respiratory infections. Brachoalveolar Lavage (BAL) fluid from calves infected with *M. haemolytica* has been shown to have elevated levels of extracellular ATP (Craddick et al. 2012). This gives credence to the theory that ATP is serving as an extracellular mediator and likely causing increased permeability through P2X7 activation. This is just one of the many possible consequences this pathway may have, as cells expressing P2X7 are found throughout the immune system, in nervous tissues, and bones. It is a safe bet that the actions of the extracellular inflammatory mediator ATP and the purinergic receptor P2X7 are just beginning to become apparent and will continue to be revealed.

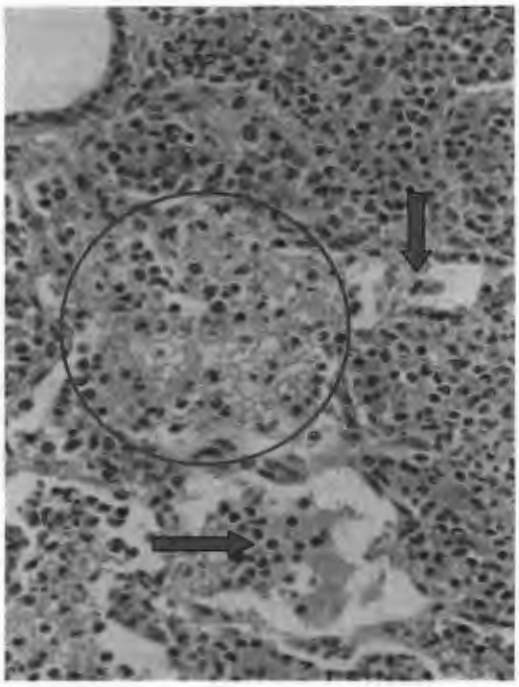

Figure 9. Alveoli Infiltration. The circle here shows an alveolus. The areas around it are usually filled with air space, but the red arrows show where leaked fluid and cells have blocked these areas, hampering function.

Future Directions of the Study

The accomplishments of this research satisfied the goals it set out to fulfill of tracking P2X7 activation and corresponding F-actin changes. However, gaps still remain to be filled and new questions are raised with its results. A variety of P2X7 antagonists highly specific to the

receptor may be used in further experiments to ensure that the results seen are strictly from activation of P2X7 and not other purinergic receptors on the cells. This may also be done by using ATP analogs which P2X7 is specifically sensitive to such as bzATP. In addition, now that the F-actin spike has been determined, it may be of use to visualize the cells during the process using confocal microscopy. Other real-time experiments during ATP exposure may include permeability test and electrical resistance testing to determine what is happening at cell junctions. As these and other tests are done, new features of the receptor may appear.

Conclusions

The results of this study show that when exposed to cells containing the P2X7 receptor, ATP causes drastic increases in the level of F-actin over a relatively short time frame (within 60 minutes). There are no indications that this is caused by anything other than activation of P2X7, and positive indicators exist in the form of greater increases between 1mM and 10mM (above the 3mM activation threshold). Though slight variation occurs, it is clear that results are repeatable with outcomes following the same trend. This data was collected on bovine MAC-T cells, but can presumably be applied to other vascular epithelial cells and any cells containing P2X7.

This evidence is important because of its link to vascular permeability changes previously discovered in cells exposed to extracellular ATP. Now that it has been confirmed that actin levels change, other conjectures and experiments can be designed around this. This especially applies in regards to actin's effect on intracellular junctions such as tight junctions and adherens junctions. Alterations in these may cause cells to pull apart and allow fluid or materials to pass through, providing a mechanism for vascular leakage and permeability. This pathway has clinical implications in Bovine Respiratory Disease Complex as well as in humans for respiratory

inflammation and possibly other areas of the body. While only a small piece of the picture, it provides useful evidence in moving forward.

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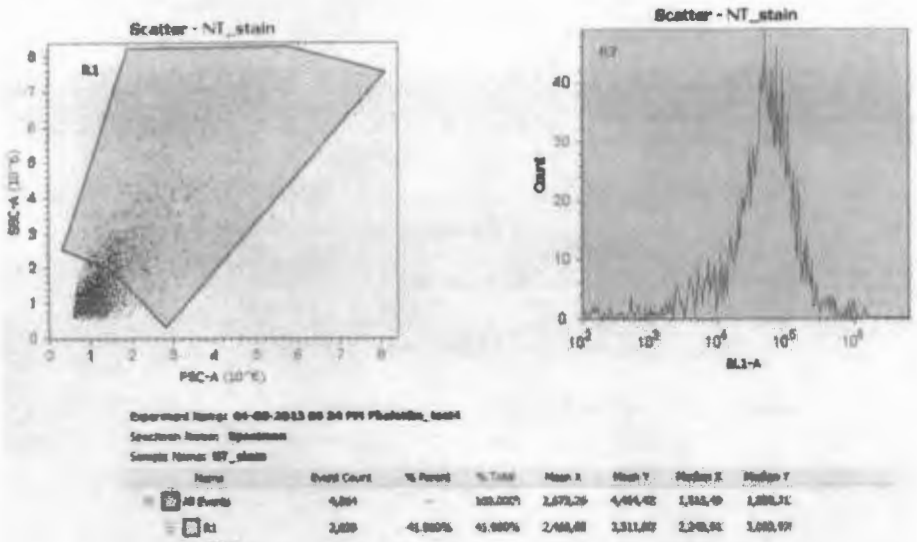
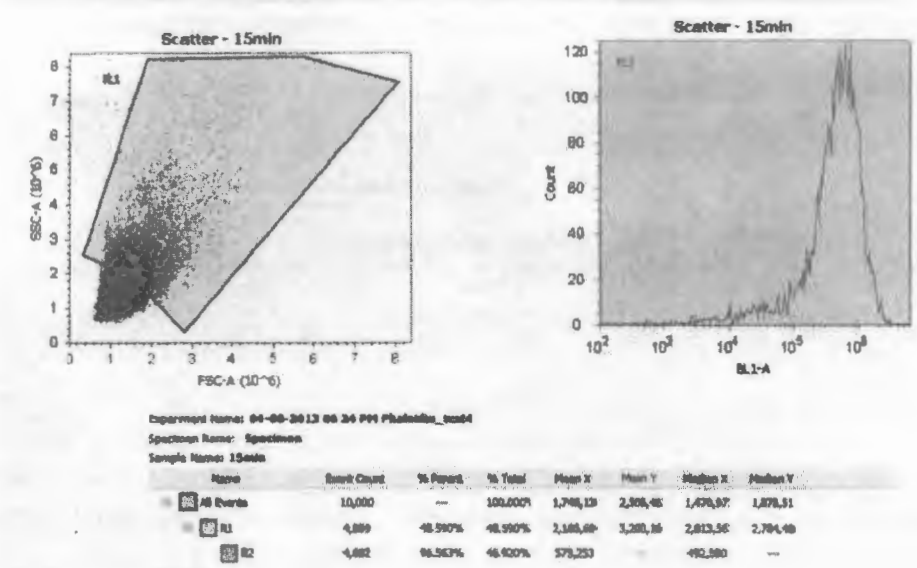
APPENDIX A- Detailed Experiment Data

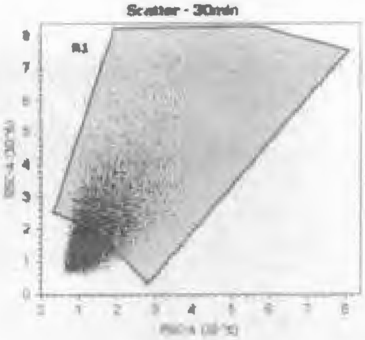
Numerical Experiment Data

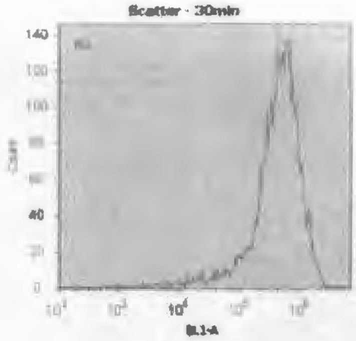
	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Mean
No Treatment	114,357	50,428	76,982	341	45,353
15 minutes	15,843	492,580	307,673	962,141	336,990
30 minutes	177,630	454,456	382,476	699,270	364,092
60 minutes	4,130*	279,631	544,792	912,732	519,659
120 minutes	675,573	212,755	440,921	114,402	348,808

Experiment 2 Flow Cytometry Display









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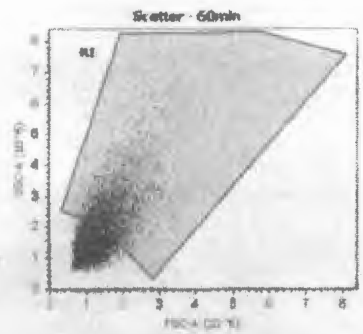
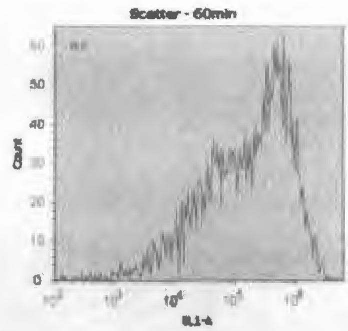
R1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
R2	[illegible]	[illegible]	[illegible]	[illegible]	—	[illegible]	—





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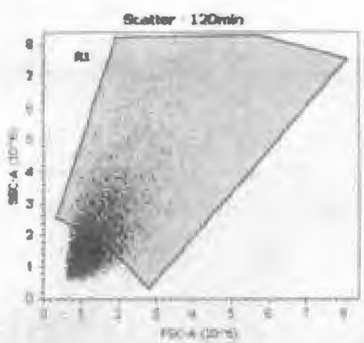
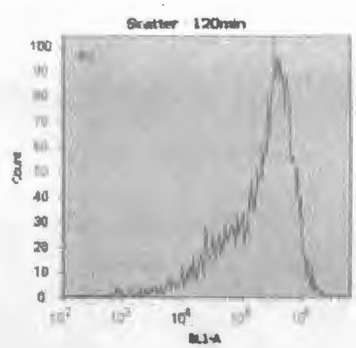
R1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
R2	[illegible]	[illegible]	[illegible]	[illegible]	—	[illegible]	—





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All Events	10,000	—	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
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R2	[illegible]	[illegible]	[illegible]	[illegible]	—	[illegible]	—