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Effects of ATP and ATP inhibitors on the P2X7 receptor

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EFFECTS OF ATP AND ATP INHIBITORS ON THE P2X7 RECEPTOR

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Abstract

Extracellular ATP has been shown to increase the permeability of cells via the P2X7 receptor. This relationship could be important in understanding the physiology of the inflammation that occurs in bovine respiratory disease complex (BRDC). My research involved testing to see if this relationship between ATP and the P2X7 receptor is present. Then, potential inhibitors of ATP were tested so they can be used in further research involving ATP and BRDC. To test bovine cells, Mac-T cells were treated with ATP and incubated for various periods of time before Yo-Pro (a fluorescent molecule) was added. The percent change in fluorescence between cells with and without ATP determined the ability ATP had in opening the P2X7 receptor pore. Results showed that extracellular ATP does bind to the P2X7 receptor to open pores. In general, the greatest change in fluorescence was seen when ATP was incubated for at least 45 minutes before the Yo-Pro was added. The actual incubation time where the greatest change in fluorescence value occurred was 60 minutes. In addition, the results from the experiments with inhibitors A were insignificant; therefore no conclusions could be made at this time.

Keywords: ATP, Yo-Pro, P2X7, Inhibitor A438079, and Inhibitor KN

Acknowledgments

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Introduction

The purpose of this research was to help address the relationship between the P2X7 receptor and its ligand, ATP. This relationship may have an important role in the physiology of bovine respiratory disease complex (BRDC), which is a very common disease affecting cattle lots. The cattle (bovine) industry supplies a large amount of beef to our food supply so our food supply depends on how well BRDC is diagnosed and treated. To be able to better diagnose and treat BRDC, we need to understand the physiology of the disease. In other words, we need to know how the disease is caused and how it thrives in the bovine body. In order to further understand the important role between the P2X7 receptor and ATP, inhibitors of the P2X7 receptor are needed to block ATP to be able to investigate the effects ATP has in inflammation occurring during BRDC, which is crucial to the success of the disease in making a bovine sick. We can compare the changes ATP makes to cells to the changes made when ATP is blocked specifically at the P2X7 receptor. Currently, inhibitors A438079 and KN62 have been used to block P2X7 receptors in humans, but they needed to be tested in bovine cells. First, it had to be determined that bovine cells actually show an increase in permeability, or leakage, caused by extracellular ATP before the inhibitors could be tested so a change could be observed between no inhibitors and the use of inhibitors.

Literature Review

Bovine Respiratory Disease Complex (BRDC), also known as shipping fever, is one of the most prominent issues in managing cattle health because it is responsible for over 50% of deaths in cattle feedlots and reduces the growth performance of cattle (Regev-Shoshani, Vimalanathan, Prema, Church, and Reudink et al., 2014). BRDC occurs as an infection of the

alveolar epithelium, which is the tissue in the air sacs of the lung (Narayanan, Kumar, Nagaraja, Chengappa, and Stewart, 2002). *Mannheimia haemolytica*, the main bacterial pathogen for BRDC, is a gram-negative coccobacillus with two main virulence factors (proteins that are on the bacteria's surface that are identified by a host's immune system as foreign object), lipopolysaccharides and leukotoxins. These factors cause apoptosis (cell death caused by the cell itself) at low concentrations and necrosis (cell death) at high concentrations in macrophages and neutrophils (immune cells) of lung tissues. Cell necrosis is associated with the airways becoming clogged with fluid and cellular debris, such as platelets and erythrocytes, from blood vessels and inflamed alveoli (Zecchinon, Fett, and Desmecht, 2005). These two processes limit proper respiratory function and can lead to death in bovines.

Recent studies have been investigating how macrophage and neutrophil cell death results in fluid leakage (caused by increased cell permeability) and inflammation in the lung. It is already known that neutrophils will activate proinflammatory cytokines, but new information has shown that leukotoxin binding can trigger signaling cascades that activate voltage-gated channels and oxygen-derived free radicals; both of which have a role in the lysis of cells and activation of immune complexes (Zecchinon et al., 2005). In addition, lipopolysaccharides increase endothelial cell cytotoxicity and neutrophil degranulation, which causes endothelial damage (McClenahan, Evanson, and Weiss, 2002). Hence, it is not the leukotoxins and lipopolysaccharides that directly cause lung damage; it is their interaction with the immune system, specifically the over activation of neutrophils and macrophages, that enables the bacteria to successfully survive in the host tissue (Zecchinon et al., 2005). Therefore, it is crucial to study the pathogenesis of this interaction to fully understand BRDC.

As mentioned previously, lipopolysaccharides and leukotoxins play roles in triggering the beginning of this pathway by activating neutrophils and macrophages, which is the pathway typical of an immune response to a foreign antigen. This eventually causes them to undergo cell death via apoptosis or necrosis. However, they do not directly cause the initial leakage of blood components into the alveoli because they do not directly increase permeability in epithelial cells (Craddick, Patel, Lower, Highlander, and Ackermann, 2012). Instead, neutrophils, macrophages, and other cells that underwent necrosis released adenosine triphosphate (ATP), the energy molecule for cells, when they were lysied, or destroyed. This extracellular ATP potentially causes an increase in cell permeability, or vascular leakage. This can allow blood components, such as platelets and immune complexes, into the airways and alveoli. Thus, ATP can be regarded as an extracellular mediator of inflammation (Craddick et al., 2012). Additionally, it has been shown that ATP increases cell permeability in bovine cells as well, specifically bovine pulmonary epithelial (BPE) cells and bovine pulmonary microvascular endothelial cells (BPMEC) by activating the P2X7 receptor (McClenahan, Hillenbrand, Kapur, Carlton, and Czuprynski, 2009).

The P2X7 receptor is homomeric, meaning several of the same subunits make up the channels, and is a type of purinergic receptor of the P2X family. This family is a class of membrane ion channels where extracellular ATP serves as the agonist, has the ability to bind and activate. In addition, high concentrations of ATP are required to activate the receptor since it is not very sensitive to ATP. Plus, the presence of divalent ions can allosterically inhibit ATP binding. The main function of P2X7 makes the cell membrane permeable to small monovalent cations and to larger, organic molecules, such as the fluorescent dye Yo-Pro (North, 2002). Actually, membrane permeability to larger molecules increases with the amount of time ATP is

bound, which can be explained by the following mechanism. When one ATP binds to a P2X7 subunit, it causes an asymmetrical orientation that deters the binding of the second ATP. In order for a second ATP to bind there must be a high concentration of ATP (more energy) and when it does bind, it opens a pore permeable to small cations. Thus, the third ATP requires even more energy to bind because the pore has even more of an asymmetrical conformation due to the binding of the second ATP. Once the third ATP binds, the pore becomes symmetric and is completely activated so that large molecules can enter the cell. (Pelegrín, 2011). However, the channel size can decrease with significantly prolonged exposure of an agonist because the receptor becomes dephosphorylated (North, 2002).

The activation of P2X7 receptor can lead to many events in the cells, including the regulation of IL-1β and IL-18, which are important inflammatory molecules, also known as cytokines. First, the receptor can let in potassium cations that activate the processing of pro-ICE to ICE (interleukin-converting enzyme), which releases mature IL-1β. Similarly, the production of IL-18 occurs by processing ICE (Lister, Sharkey, Sawatzky, Hodgkiss, Davidson et al., 2007). It is important to note that these pathways have not yet been confirmed in animal species. These cytokines then activate the innate immune system, which is responsible for inflammation. In addition, it has been demonstrated that extended activation of P2X7 can cause apoptosis in mast cells and epithelial cells. Rapid apoptosis can lead to secondary necrosis, which then leads to even more inflammation (Lister et al., 2007). Furthermore, the P2X7 receptor is located on many haemopoietic cells, including macrophages and neutrophils (Lister et al., 2007). When lipopolysaccharides and leukotoxins trigger necrosis of cells in the lung tissue, ATP is released and activates the P2X7 receptor of nearby cells; which then causes inflammation and increased permeability via the various signaling cascades in the lung tissues that have been discussed.

There are five main categories of antagonists (blockers) for P2X7 receptor including: ions (calcium, magnesium, zinc, copper, and protons), generic antagonists (Brillant Blue G and oxidized ATP), large organic cations (calmidazolium and KN-62), 17β-estradiol, and some monoclonal antibodies. The relevant category to this study is the large organic cations, specifically KN-62 (a piperazine), which inhibit the P2X7 receptor reversibly and is voltage independent. Currently it is used to block currents via the P2X7 receptor in rats, but it does not seem to do the same in humans (North, 2002). Inhibitor A438079 has been used in many studies for the purpose of blocking ATP and BZ-ATP to study the P2X7 receptor, such as in murine macrophage cells (Guadalupe, 2010).

As mentioned previously, the P2X7 receptor is capable of letting in certain dyes, such as ethidium and Yo-Pro. Dyes are useful in studying P2X7 because they fluoresce when they encounter nucleic acids within the cell. Then, their measured fluorescence can be correlated to how much dye entered the cell and thus indicate the degree of dilation in the receptor pore (or in other words, how permeable the cell is). However, some studies show that Yo-Pro takes a longer time to enter the pore of the P2X7 receptor compared to a normal ionic current in physiological conditions. These same studies indicate that there may be species differences in the uptake of Yo-Pro and even the blocking capabilities of antagonists (North, 2002). Therefore, it is important to study the effects of these molecules specifically in bovine cells to know whether their effects are similar or different to existing knowledge.

Materials and Methodology

To determine whether bovine cells, specifically Mac-T cells, increase in permeability in response to extracellular ATP, an experiment needed to be set up in a way that fluorescence

values, determined by the amount of Yo-Pro entering the cells via the P2X7 receptor pore, could be compared between cells with ATP and cells without ATP. The negative controls had Yo-Pro, but no ATP in order to see the baseline fluorescence (fluorescence that was not caused by ATP) and this was compared to the fluorescence of the positive controls, which had Yo-Pro and ATP. The positive control was predicted to increase in fluorescence due to the addition of ATP, which would open the P2X7 pore and let in more Yo-Pro to fluoresce. The ATP needed to be incubated to give it time to open the P2X7 pores. The first set of experiments made the incubation time the variable to see how long of an incubation was required to get maximum fluorescence. This incubation time was used in the second set of experiments, which were designed to test how well the inhibitors could eliminate or reduce fluorescence by blocking ATP from binding to P2X7 so Yo-Pro could not enter. To compare how well the inhibitors reduced fluorescent, cells with ATP, Yo-Pro, and the inhibitors were compared to positive control cells containing just Yo-Pro and ATP. It was predicted that the cells with the inhibitors would have lower fluorescence values than positive control, the cells with no inhibitors.

Mac-T Cells

Cell media was made using DMEM with 10% fetal bovine serum, 1% L-glutamine, and 1% antibiotics (penicillin/streptomycin). The Mac-T cell line, an immortalized bovine mammary gland endothelial cell line, was used for the experiments. Five vials of stock cells were kept in liquid nitrogen as back up cells to use when contamination occurred. When they were needed, a vial was thawed in a warm bath for about two and a half minutes. Once the cells were started in T-25 flasks, they were transferred to new T-25 flasks with fresh media once a week to ensure they kept growing and stayed healthy. One flask from every new cell passage was left in the

incubator as a back-up and the previous reserve flask was thrown away in a biohazard container. Of the other two flasks, one was split into three new flasks and one was used for an experiment. To split a flask, the old media was removed and the flask was rinsed with sterile PBS to remove any remaining old media. Then, trypsin was added to the flasks, to detach the cells from the flask surface, and it was then put in the incubator for five minutes. Once 80% of the cells detached, 2mL of media was added to stop the trypsin reaction. The sides of the flask were rinsed off using the media in the flask before the media was transferred to a 15mL conical tube and centrifuged for five minutes at 3300 rpms. The old media was descanted into a waste beaker with 12mL of fresh media taking its place and the cells were re-suspended. Then, the media was aliquoted to new flasks. The flasks were stored in the incubator at 38˚ C.

Inhibitor Stock and Working Solutions

The inhibitors came in a powdered form and needed to be made into stock solutions and working solutions. A 0.05M stock solution of inhibitor A438079 hydrochloride (inhibitor A) was made by adding 292 mL DMSO to the 10 mg sample of inhibitor A in its original container. Then, 2µL of inhibitor A stock solution was diluted with18 µL of PBS in a small tube to make a 0.005M working solution. Likewise, a 0.05M stock solution of inhibitor KN (inhibitor K) was made by adding 13.9 µL of DMSO to 1mg sample of inhibitor K in its original container. Then, 1µL of inhibitor K stock solution was diluted with 999µL PBS to make a 0.05mM working solution. The stock solutions were stored in a freezer at -80 degrees Celsius and the working solutions were stored in a refrigerator.

Yo-Pro and Triton Working Solutions

The Yo-Pro was kept in the freezer at 0˚ C and was allowed to de-thaw in a dark place so it did not lose its fluorescence. The working solution consisted of 10uL Yo-Pro and 90uL PBS to make it 1.0µM solution. The working solution was stored in the refrigerator.

ATP Solutions

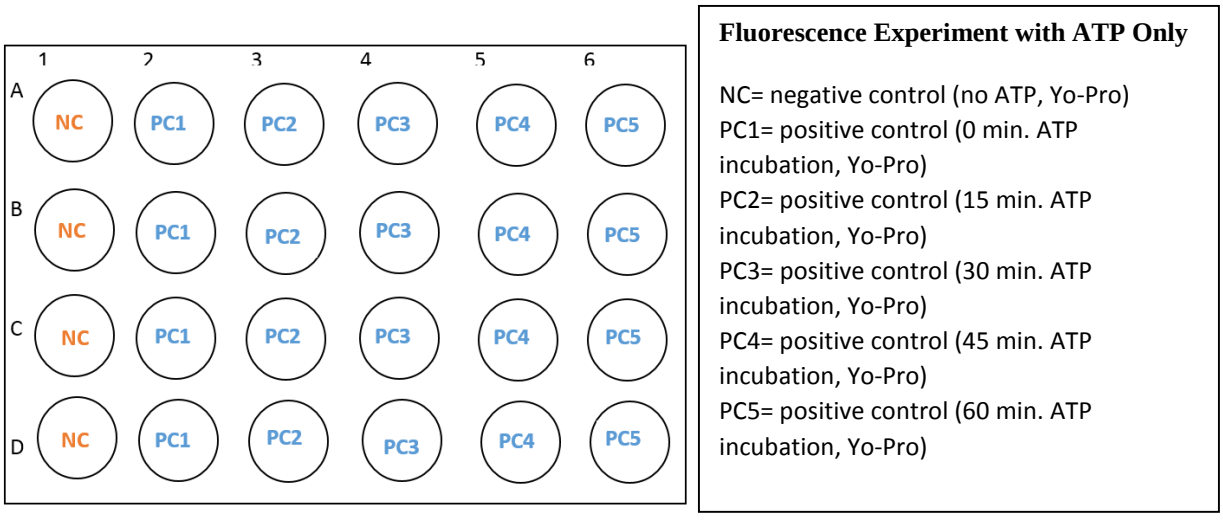
An ATP solution was made out of the powdered ATP. 0.276g of powdered ATP, stored in the freezer, was added to 1mL distilled water to make a 0.5M solution. The pH was adjusted to a pH range of 6.2-6.8 by adding 0.1M NaOH. Then, the ATP solution was aliquoted to vials and stored in the -80 degree freezer until it was needed.

Fluorescence Experiment with ATP Only

Mac-T cells from a confluent T-25 flask were re-suspended in 12 mL of media using the same procedure used in splitting the cells. However, instead of the media cell solution being divided into three new flasks, it was portioned out into a 24 well plate with 0.5mL cell media per well. The cells were incubated at 38˚ C until confluent; which took between two and three days. Once they were confluent, the old media was dumped out and the plate was rinsed with 300µL PBS twice. ATP was incubated for varying time periods of 0, 15, 30, 45, and 60 minutes before Yo-Pro was added. These were labeled as PC1 (positive control 1), PC2, PC3, PC4, and PC5 respectively (figure 1). Readings were taken every 15 minutes using a fluorescent plate reader for 90 minutes after each initial ATP incubation period. 0.4µL ATP, to make it 1mM ATP, (ATP was allowed to de-thaw from -80 degree Celsius freezer before addition) was added to columns two through six, which was all the PC columns. 2.0 µL Yo-Pro was only added to column one,

negative control (NC) and two (PC1) as shown in figure 1. Immediately, the plate was placed in the fluorescence machine, which was set up using the Yo-Pro programming set to read at a wavelength of 390nm. The fluorescence of the NC and PC1 was recorded under read time labeled 0 minutes. After the first read, the plate was put back in the incubator. After 15 minutes, 2.0 µL Yo-Pro was added to column three (PC2) and placed in the fluorescence machine. The fluorescence of the NC, PC1, and PC2 were recorded under a read time labeled 15 minutes and 0 minute ATP incubation for the NC and PC1 and a read time labeled 0 minutes and 15 minute incubation for PC2. The plate was then incubated for another 15 minutes. Additional 2.0 µL of Yo-Pro was added every 15 minutes to a new positive control column followed by a read (of all the columns containing Yo-Pro). This continued until PC5 (in the last column, six) received Yo-Pro. Then, the plate was left in the incubator where it was read every 15 minutes for another two hours. Therefore, fluorescence readings were taken at time points 0, 15, 30, 45, 60, 75, and 90 minutes for the negative control and each positive control with different ATP incubation times (0, 15, 30, 45, and 60 minutes).

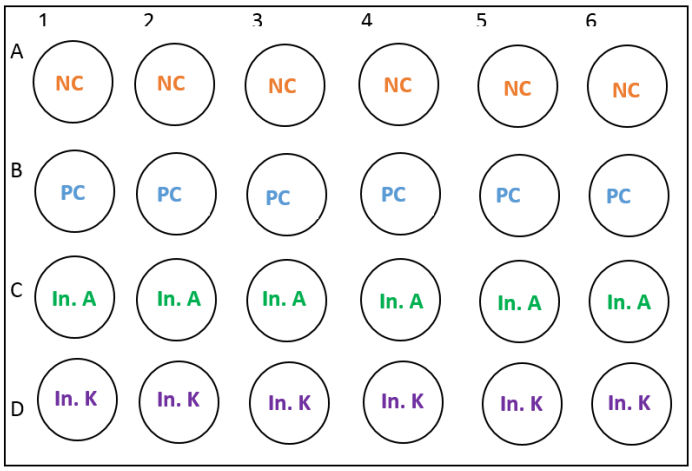
Figure 1



Fluorescence Experiment with ATP Inhibitors

Mac-T cells from a confluent T-25 flask were re-suspended in 12 mL of media using the same procedure used in splitting the cells. However, instead of the media cell solution being divided into three new flasks, it was portioned out into a 24 well plate with 0.5mL cell media per well. The cells were incubated at 38 degrees Celsius until confluent (little to no space between the cells); which took between two and three days. Once they were confluent, the old media was dumped out and the plate was rinsed with 300µL PBS twice. Then, 200uL of PBS (at room temperature) was added to each well and 0.4µL of inhibitors A438079 and KN was added to rows C and D respectively (figure 2). Then, the plate was incubated for 15 minutes. During that time the ATP was allowed to de-thaw. After the 15 minute incubation, 0.4µL ATP (to make it 1mM ATP) was added to columns B, C, and D (figure 2) and the plate was incubated again for an hour. After the second incubation, 2.0 µL Yo-Pro was added to every well. Immediately, the plate was placed in the fluorescence plate reader, which was set up using the Yo-Pro programming set to read at a wavelength of 390nm and record the fluorescence every 15 minutes for one hour.

Figure 2



Fluorescence Experiment with ATP and Inhibitors

NC= negative control (no ATP, Yo-Pro)
PC= positive control (only ATP)
In. A= inhibitor A438079, ATP, Yo-Pro
In. K= inhibitor KN, ATP, Yo-Pro

Data Analysis

In order to compare the effect of different ATP incubation periods, the data needed to be normalized. This was done by evaluating the positive controls against the negative control to find the change in fluorescence that the ATP caused. The change in fluorescence was calculated by dividing the positive control (Yo-Pro and ATP) fluorescence values by the negative control (Yo-Pro and no ATP) fluorescence values. The fluorescence values for each read time for each positive control were divided by the negative control fluorescence value at the same read time as the positive control being examined. For instance, the fluorescence value at read time 30 minutes for PC1 was divided by the fluorescence value for the NC at its read time at 30 minutes. The change in fluorescent values for each read time point of each positive control were averaged across the three experiments to come up with a final table and graph comparing the change in fluorescence caused by various ATP incubation periods. This data was used to determine the incubation and read time for the fluorescence experiment with ATP and inhibitors. Similarly, the data from the fluorescent experiment with ATP and inhibitors was normalized and averaged in the same manner. The positive control, inhibitor A, and inhibitor K were each divided by the negative control at the same time point. Then the averages of each time point were calculated across the five experiments.

The normalized values from the fluorescent experiment with ATP were analyzed by ANOVA to determine statistical significance, using a p-value in a 95% confidence interval, of the data set as a whole. The Scheff F-test was used to determine if significant differences existed between each incubation period. Also, the normalized values from the fluorescence experiment with ATP and inhibitors were analyzed using ANOVA to determine statistical significance using a p-value in a 95% confidence interval.

Results

Fluorescent Experiment with ATP Only

After running the results through an ANOVA analysis, a p-value of 0.0001 was obtained, meaning the data is statistically significant. As shown in Figure 3a, the greatest change in fluorescence was 1.954 and that was after a 60 minute ATP incubation period with a read time at 60 minutes. In fact, the 60 minute ATP incubation period gave the greatest overall change in fluorescence regardless of the read time, with one exception at the initial (0 minute) read time where it was the second. The 45 minute ATP incubation time gave the second greatest overall change in fluorescence regardless of the read time, except at the initial read time where it was the greatest, with the highest change being 1.880. In general, the 45 and 60 minute ATP incubation times had much greater changes in fluorescence than the 0, 15, and 30 minute ATP incubation times regardless of the read time (except at the initial read time where the changes in fluorescence values were closer). The Scheffe F-test indicated significant differences in the change of fluorescence between the 45 minute ATP incubation period and the 0, 15, and 30 minute ATP incubation periods (Figure 4). Likewise, there is a significant difference in the change of fluorescence between the 60 minute ATP incubation period and the 0, 15, and 30 minute ATP incubation periods (Figure 4). There is no significant difference (0.011) between the 45 and 60 minute ATP incubation times.

Change of fluorescent values less than 1 mean that there was a decrease in fluorescence with the addition of ATP. This occurred once at the 0 minute read time point, where it was 0.968. Further, the 0 minute ATP incubation time had the lowest changes for five of the seven read times with it only ever reaching 1.234. However, the 0, 15, and 30 minute ATP incubation periods were similar in their change of fluorescence, having no significant differences (Figure 4).

Looking strictly at the read time points for every incubation period, the greatest change takes place between the 0 and 30 minute read times, then after that it stays about the same in the degree of change in fluorescence between read time points.

Figure 3a

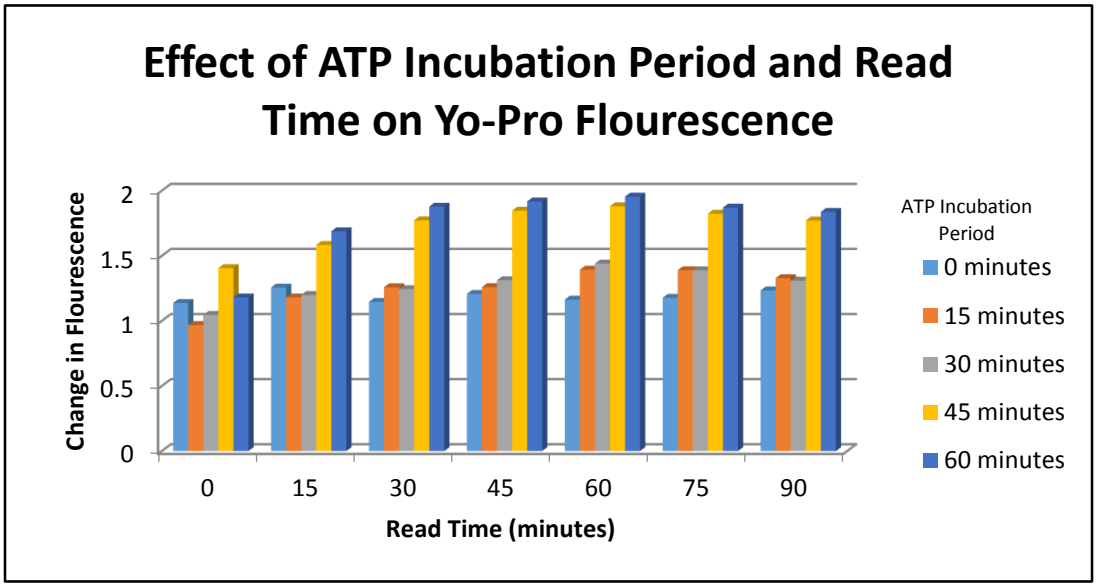


Figure 3b

Data Table for Effect of ATP Incubation Period and Read Time on Yo-Pro Fluorescence

		ATP Incubation Period (minutes)				
		0	**15**	**30**	**45**	**60**
Read Time (minutes)	**0**	1.137	0.968	1.046	1.405	1.18
	15	1.256	1.18	1.199	1.583	1.688
	30	1.146	1.258	1.244	1.772	1.877
	45	1.207	1.259	1.312	1.846	1.917
	60	1.163	1.393	1.44	1.88	1.954
	75	1.177	1.389	1.389	1.823	1.87
	90	1.234	1.328	1.309	1.77	1.837

Figure 4

Comparison of Entire ATP Incubation Periods

ATP Incubation Period	*Mean*	*Standard Error*
0	1.188	0.035
15	1.254	0.037
30	1.279	0.034
45	1.735*	0.158
60	1.76*	0.102

*difference with 0, 15, and 30 period determined significant at 95% by Scheffe F-test

Fluorescent Experiment with ATP and Inhibitors

After running the results through an ANOVA analysis, a p-value of 0.8573 was obtained, meaning the data is statistically insignificant. This was expected after graphing the data obtained from the experiment. As seen in figure 5, there is little to no difference between the control and each of the inhibitors. Additionally, there is little to no difference between the read times for the control, inhibitor A, and inhibitor K. Even though there is no significance, some trends can be seen. Inhibitor A is consistently greater than the positive control and inhibitor K is lower than the positive control in the last three read times.

Figure 5a

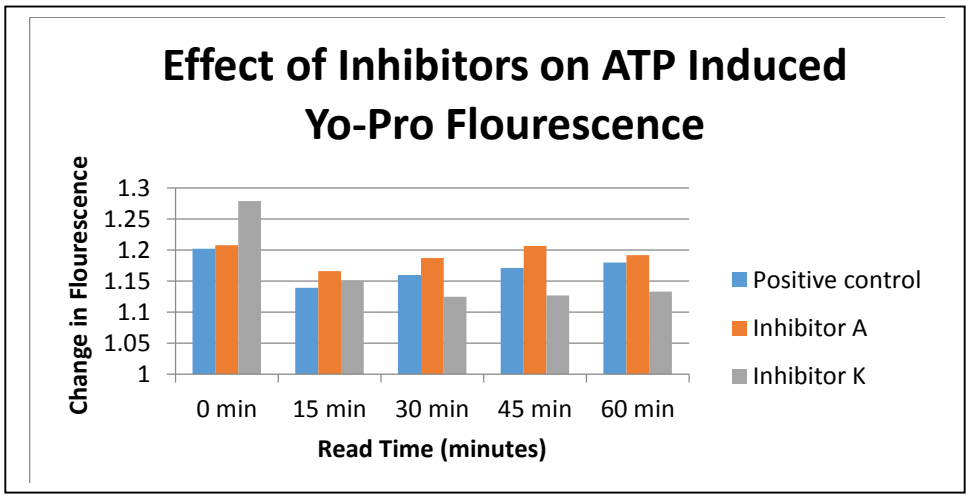


Figure 5b

Data Table for Inhibitors on ATP Induced Yo-Pro Fluorescence

	0 min	*15 min*	*30 min*	*45 min*	*60 min*
Positive control	1.202	1.139	1.16	1.171	1.18
Inhibitor A	1.208	1.166	1.187	1.207	1.192
Inhibitor K	1.279	1.151	1.125	1.127	1.133

*significant at 95%

Discussion

Fluorescent Experiment with ATP Only

The experiment's purpose was to determine the optimal fluorescence that can be achieved with the right ATP incubation period and read time combination so more information can be known about the interaction between ATP the P2X7 receptor. Plus, the results served as a guideline for setting up the protocol to test inhibitors of ATP because the ATP incubation period and read time with the highest fluoresce needed to be used to make sure the inhibitor can block ATP at ATP's peak effectiveness. From the results, it shows that the optimal fluorescence occurs after a 60 minute ATP incubation period and a 60 minute read time. This means that it takes a total of 120 minutes for ATP to bind to the P2X7 receptor in a manner that allows the maximum amount of Yo-Pro to enter the cell. In general, the results show an increase in fluorescence as the ATP incubation period increases, meaning it takes a certain amount of time for ATP to open

P2X7's pore. This supports previous evidence claiming that there is a time-dependent increase in permeability. Specifically, a new model proposes that P2X7's pore expands as more ATP binds to it. Without ATP, the P2X7 receptor is closed, symmetrical, and has a high affinity for ATP, causing ATP to bind when it is around. Once one ATP is bound, a conformation change happens so the receptor becomes asymmetrical and has a low affinity for ATP (making it hard for more ATP to bind). When the second ATP does bind, it causes another conformational shift and lowers the affinity for ATP even more. Thus, it takes a lot to get the third ATP bound to the receptor. When it does, the receptor becomes symmetrical again and the new conformation has a wide enough pore for large molecules, like Yo-Pro, to go into the cell (Pelegrín et al., 2011). Based on the results obtained that fluorescence increases with increased ATP incubation (meaning more pores are open after a longer ATP incubation period), it is possible this is the mechanism taking place in bovine Mac-T cells. There were no significant changes in fluorescence for the first 30 minutes of incubation, meaning this is when the first two ATP's were binding to the receptor because most of the pores were still closed so not as much Yo-Pro could enter cells to fluoresce. Once the third ATP had time to bind, there was a significant change in fluorescence (resulting from much more Yo-Pro entering the cell because more pores were open), which occurred during the 45 and 60 minute incubation periods (when the third ATP was given enough time to bind to the receptor). The 30 minute or less incubation times were not enough time for the majority of the P2X7 receptors to gain that third ATP, which is why the 0, 15, and 30 minute incubation periods had much lower fluorescence values than the 45 and 60 minute incubation periods. In addition, the fluorescence correlates with the cell's permeability because more fluorescence is a result of more Yo-Pro entering the cell, which means the P2X7 pore is open. The cell is more permeable when the pores are open. Yo-Pro entering the cell in

vitro (in a laboratory setting) represents possible biological factors and ions that could enter or leave a cell in vivo (in a body), which could have a part in regulating inflammation. Inflammation causes many of the problems associated with BRDC, including swelling in the lung tissues and clogging of the airways with fluid and cellular debris. By knowing the mechanism of action of ATP and how it leads to inflammation is crucial in the path to being able to better treat and diagnose inflammatory injuries and diseases, including BRDC.

The additional important piece of information from this data is that there is no significance in the read time. In other words, it does not matter when the plate is read after its ATP incubation. The length of ATP incubation is more important because it showed significant changes. One possible reason the read time isn't significant, is because the concentration of ATP does not change. Once all of the ATP has been used to bind the P2X7 receptors, there will not be anymore left to be used in opening more pores so no more Yo-Pro can enter and increase fluorescence. This is most likely the case with the 45 minute and 60 minute ATP incubation periods were maximum fluorescence is reached, meaning all the ATP is used. However, this does not have much importance related to inflammation because in vivo, the ATP concentration will not be limited to a certain amount. In the 0, 15, and 30 minute ATP incubation periods, not all of the ATP is used to bind P2X7 receptor pores to let in Yo-Pro to fluoresce. This is known because the fluorescence values are not as high as the 45 and 60 minute ATP incubation periods. Therefore, it is possible the addition of Yo-Pro somehow limits the rest of the unbound ATP in binding to the receptor pore because theoretically ATP should continue to bind to the receptor pore even after Yo-Pro has been added. This would mean a significant increase in fluorescence would be seen after 60 minutes of the ATP being with cells regardless of when Yo-Pro was added.

Furthermore, the initial read time (0 minutes) does not follow the same pattern as the rest of the read times (which is the longest incubation period at each time point having the highest change in fluorescence). This is mostly likely due to variance between experiments caused by the user. The initial read is supposed to be taken immediately once the Yo-Pro is added, but the time it takes to add the Yo-Pro and add the plate to the fluorescence reader can vary between experiments.

Fluorescent Experiment with ATP and Inhibitors

The data from this experiment was determined not to be significant so no definite conclusions can be made. One reason the data is not significant is because the positive control did not show a large increase in fluorescence compared to the negative control, as it had previously done with ATP only. Therefore, it makes it difficult to determine whether the inhibitors were working or not because there is not a good control to compare them to. Work is currently being done to determine why the Mac-T cells were no longer behaving like they did in the initial experiment without inhibitors, including looking at the cell passage number of the cells (age) and the growth environment. However, there is a trend that is worth noting and to watch for in further experiments. Inhibitor A was consistently greater than the positive control, which means it did not block the ATP from binding to the P2X7 receptor. Also, inhibitor K had fairly consistent values that were less than the positive control, meaning that the inhibitor KN could potentially have an antagonist affect on ATP; although, more testing needs to be done to make definite conclusions.

Conclusion

The research conducted was aimed at trying to answer more questions about the mechanism that explains the interaction between ATP and the P2X7 receptor. More specifically, this research looked at ATP's ability to increase a cell's permeability by binding the P2X7 receptor and opening its pore. Yo-Pro was used to measure the increase in permeability based on its ability to fluoresce once it enters a cell via the P2X7 receptor pore. The fluorescence was proportional to the amount of Yo-Pro in the cell and the cell's permeability. The more fluorescence means more Yo-Pro, which proves more pores are open and the cell is more permeable. Using this logic, the relationship between the P2X7 pore and its agonist, ATP, could be analyzed based on time (in other words, how long it takes ATP to open the P2X7 receptor pore). The results showed that an incubation time period of 60 minutes gave the highest fluorescence, which means it took 60 minutes for enough ATP to bind and open the P2X7 pore to let in the fluorescent Yo-Pro. This supports the proposed mechanism of opening the P2X7 pore, which is that the pore has to undergo three, energy-dependent (ATP) conformational changes before it can open. It has to have three ATP bound before it is able to let in Yo-Pro and this takes between 45 and 60 minutes based on the results this research found. By knowing that the receptor pore goes through a slow conformational change that is dependent on the number of ATP bound to it, the beginning processes of inflammation can be more understood.

Additionally, potential inhibitors of ATP were tested in their abilities to block ATP from binding to the P2X7 pore and thus reducing inflammation. The same logic as before was applied here. If ATP with inhibitors caused a lower fluorescence than ATP only, then that meant the inhibitors were able to block ATP so ATP couldn't open the pore and let in Yo-Pro. The results obtained from this research were statistically insignificant, but a very weak positive control was to blame. Therefore, it cannot be concluded whether the inhibitors were effective because the

positive control to compare them to was insufficient. It is important to continue trying to test the inhibitors to determine if they have the capabilities to block ATP. They have a very important use in continuing to acquire knowledge about the interaction of ATP and inflammation. The inhibitors can be used to block ATP to determine what effects it has in the inflammation pathway, specifically in connection with inflammation in the lung that occurs in BRDC. Research comparing structural changes in the cell with ATP and with blocked ATP could be very useful in determining the importance of blocking extracellular ATP as a treatment option.

Moreover, this research did have some limitations that made it difficult to analyze. The biggest limitation was not having consistent cell lines between experiments that would yield a sufficient positive control to use for comparisons. Many times the positive control would have a high fluorescence one week, which was ideal, and then it would be low the next week. Factors causing this fluctuation could not be determined, but factors that were examined include: cell line type, cell passage number, age of cells when the experiment was conducted, and age of ATP.

Overall, the additional information gathered about ATP in this research can be added to the existing knowledge about ATP and inflammation to help other researchers studying this topic. Learning more about inflammation, even small amounts, brings us closer to developing better diagnostics and treatments. In terms of BRDC, this would reduce costs in the cattle industry related to mortality and low cattle weights, caused by illness. With more cattle, and higher quality of cattle, going to market, more beef will be produced to supply our world's food demands.

Appendix

Fluorescence Experiment with ATP Only

Normalized Data

Experiment #30

Read Time (minutes)	0	15	30	45	60
0	0.9531	1.051	1.189	2.221	1.19
15	1.241	1.288	1.223	2.439	2.283
30	1.015	1.19	1.209	2.826	2.386
45	1.126	1.346	1.424	2.936	2.459
60	1.039	1.482	1.408	2.889	2.472
75	1.056	1.39	1.271	2.792	2.45
90	1.064	1.375	1.306	2.732	2.261

Experiment #34

Read Time (minutes)	0	15	30	45	60
0	1.36	1.001	1.119	1.156	1.432
15	1.366	1.168	1.168	1.234	1.39
30	1.412	1.247	1.324	1.266	1.815
45	1.453	1.146	1.238	1.476	1.904
60	1.377	1.182	1.549	1.516	1.974
75	1.311	1.311	1.545	1.524	1.974
90	1.443	1.223	1.39	1.445	1.922

Experiment #35

Read Time (minutes)	0	15	30	45	60
0	1.097	0.851	0.829	0.838	0.0918
15	1.16	1.085	1.202	1.075	1.39
30	1.01	1.336	1.198	1.225	1.429
45	1.043	1.286	1.274	1.127	1.388
60	1.072	1.516	1.362	1.236	1.417
75	1.162	1.466	1.351	1.153	1.39
90	1.162	1.466	1.351	1.153	1.39

Raw Data

Experiment #30

	ATP Incubation Period (minutes)					
Read Time (minutes)	0	15	30	45	60	NC
0	176.8	195	220.5	412	221	185.5
15	227.5	236	224.8	447	418.5	183.3
30	187.5	220	223.5	522.3	441	184.8
45	201.5	241	225	525.5	440	179
60	186.5	266	252.8	518.5	443.8	179.8
75	208.8	275	251.5	552.3	444	197.8
90	208.8	276	256.3	536.3	443.8	196.3

Experiment #34

	ATP Incubation Period (minutes)					
Read Time (minutes)	0	15	30	45	60	NC
0	183.3	135	150.8	155.8	193	134.8
15	183	137	156.5	165.3	186.3	134
30	185	163.3	173.5	165.8	237.8	131
45	190.8	150.5	162.5	193.8	250	131.3
60	177.3	152.3	199.5	195.3	257.8	128.8
75	175	175	206.3	203.5	263.5	133.5
90	207	175.5	199.5	207.3	275.8	143.5

Experiment #35

	ATP Incubation Period (minutes)					
Read Time (minutes)	0	15	30	45	60	NC
0	198.8	154.3	150.3	152	166.5	181.3
15	163.3	152.8	169.3	151.3	196	140.8
30	144.5	161	171.3	175	204.3	143
45	156	192.3	190.5	168.5	207.5	149.5
60	151	213.5	191.8	174	199.5	140.8
75	171.5	216.3	190.3	170	205	147.5
90	171.5	216.3	190.3	170	205	147.5

Fluorescence Experiment with ATP and Inhibitors

Normalized Data

Experiment #41

	0 min	15 min	30 min	45 min	60 min
PC	1.688	1.308	1.355	1.38	1.372
A	1.377	1.201	1.206	1.307	1.265
K	1.173	1.039	1.045	1.028	1.07

Experiment #43

	0 min	15 min	30 min	45 min	60 min
PC	0.948	1.095	1.157	1.172	1.166
A	1.267	1.352	1.407	1.34	1.29
K	1.889	1.62	1.555	1.508	1.43

Experiment #44

	0 min	15 min	30 min	45 min	60 min
PC	1.082	1.085	1.041	1.037	1.07
A	1.063	1.044	1.029	1.082	1.105
K	1.139	0.948	0.897	0.939	0.938

Experiment #45

	0 min	15 min	30 min	45 min	60 min
PC	1.128	1.158	1.117	1.178	1.184
A	1.219	1.208	1.201	1.203	1.262
K	1.087	1.155	1.103	1.108	1.187

Experiment #46

	0 min	15 min	30 min	45 min	60 min
PC	1.163	1.048	1.126	1.089	1.106
A	1.112	1.024	1.091	1.104	1.04
K	1.109	0.994	1.024	1.051	1.041

Raw Data

Experiment #41

	0 min	15 min	30 min	45 min	60 min
NC	135.7	145.3	147.8	161.5	167.3
PC	229	190	200.3	223	229.5
A	186.8	174.5	185.8	211	211.7
K	159.2	150.8	154.5	166	179

Experiment #43

	0 min	15 min	30 min	45 min	60 min
NC	178.2	189	194.5	209	223
PC	169	207	225	245	260
A	225.8	255.5	273.7	280	287.7
K	336.7	306.2	302.5	315.2	318.8

Experiment #44

	0 min	15 min	30 min	45 min	60 min
NC	158	175.5	183.7	183.3	185.2
PC	171	192.2	191.2	190	198.2
A	168	183.2	189	189.3	204.7
K	180	166.3	164.7	172.2	173.8

Experiment #45

	0 min	15 min	30 min	45 min	60 min
NC	149	159.8	168.5	171.8	173.5
PC	168	185	188.2	202.3	205.5
A	181.7	193	202.3	206.7	219
K	162	184.5	185.8	190.3	206

Experiment #46

	0 min	15 min	30 min	45 min	60 min
NC	137	165	164.3	168.2	169.5
PC	160.3	173	185	183.2	187.5
A	153.2	169	179	185.7	176.3
K	152.8	164	168.2	176.8	176.5

Literature Cited

Craddick, M., Patel, R., Lower, A., Highlander, S., Ackermann, M., and McClenahan, D. 2012. "Adenosine-5'-Triphosphate Release by Mannheimia Haemolytica, Lipopolysaccharide, and Interleukin-1 Stimulated Bovine Pulmonary Epithelial Cells." *Veterinary Immunology and Immunopathology* 149 (1-2): 58–65.

Guadalupe M., Casas-Pruneda, G., Ortega, F., Sanchez-Armass, F., Perez-Cornejo P. and Arreola, J.. 2010. "Stimulation of P2X7 receptors causes Ca2+- and PKC-mediated reactive oxygen species production in murine macrophages" *The FACEB Journal* 28(10)

Lister, M., Sharkey J., Sawatzky, D., Hodgkiss, J., Davidson, D., Rossi, A., and Finlayson, K.. 2007. "The Role of the Purinergic $P2X_7$ receptor in Inflammation." *Journal of Inflammation* 4 (5): 1186-1476.

McClenahan, D., Hillenbrand, K., Kapur, A., Carlton, D., and Czuprynski, C. 2009. "Effects of Extracellular ATP on Bovine Lung Endothelial and Epithelial Cell Monolayer Morphologies, Apoptoses, and Permeabilities." *Clinical and Vaccine Immunology: CVI* 16 (1): 43–48.

McClenahan, D., Evanson, O., and Weiss, D. 2002. "In Vitro Evaluation of the Role of Platelet-Activating Factor and Interleukin-8 in Mannheimia Haemolytica-Induced Bovine Pulmonary Endothelial Cell Injury." *American Journal of Veterinary Research* 63 (3): 394–401.

Narayanan, S., Nagaraja, T., Chengappa, M., and Stewart, G. 2002. "Leukotoxins of Gram-Negative Bacteria."*Veterinary Microbiology* 84 (4): 337–56.

North, Alan R., 2002. "Molecular Physiology." *Physiological Reviews* 82 (3):1013-1067.

Pelegrín, P. 2011. "Many Ways to Dilate the P2X7 Receptor Pore."*British Journal of Pharmacology* 163 (5): 908–11.

Regev-Shoshani, G., Vimalanathan, S., Prema, D., Church, J., Reudink, M., Nation, N., and Miller, C. 2014. "Safety, Bioavailability and Mechanism of Action of Nitric Oxide to Control Bovine Respiratory Disease Complex in Calves Entering a Feedlot." *Research in Veterinary Science* 96 (2): 328–37.

Zecchinon, L, Fett, T., and Desmecht, T. 2005. "How Mannheimia Haemolytica Defeats Host Defence through a Kiss of Death Mechanism." *Veterinary Research* 36 (2): 133–56.