Mechano-response via ATP alters calcium signaling in breast epithelial cells with oncogenic KRas mutation

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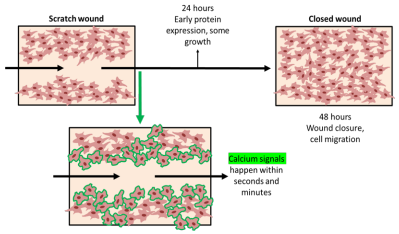
Abstract

The majority of breast cancer patient deaths occur when tumor cells migrate away from the primary tumors and disseminate metastatically. Cancer cells at the invasive front mimic the behaviors of non-tumorigenic epithelial cells at wound edges but show less coordinated migration comparatively. While most wound healing studies use a timeframe of 24-48 hours, our previous studies have shown that ATP released from wounded cells initiates a calcium (Ca2+) signal within seconds that spreads to neighboring cells through activation of the P2Y2 surface receptor. RNAseq data of MCF10A mammary epithelial cells with stepwise mutations of PTEN knockout and KRas activation alone or in combination, demonstrate that P2Y2 is downregulated specifically by active KRas. Fluorescence microscopy was used to measure Ca2+ signaling and mitochondrial membrane potential in parental and mutant MCF10A cells. Change in total fluorescence was measured and compared to baseline using Fluo-4, a Ca2+ fluorescent indicator, and TMRM (, methyl ester), a fluorescent mitochondrial membrane potential dye on Nikon analysis software. We show that KRas activation disrupts ATP stimulation and Ca2+ signaling. This change was further quantified using a FlexStation III plate reader with Fluo-4 to read specific fluorescent changes in Ca2+ after ATP addition. ATP-stimulated Ca2+ was also disrupted in other breast tumor cell lines harboring Ras activating mutations, MDA-MB-231 and MDA-MB-436, both showing a similar decrease in P2Y2 expression. These data show that ATP stimulation can be used to further understand the differences in Ca2+ signaling and mechanical-response between normal breast epithelial cells and cancer cells. Clarifying these molecular mechanisms could reveal targets for new cancer treatments, especially since 90% of human tumors are epithelial carcinomas.



Introduction and Background

- Scratch wound assays are typically studied at 24-48 hours in cancer biology
- Calcium signals in similar pathways occur within seconds to minutes, as found in cardiac, muscle, and bone studies
- Previous work in our lab has show that scratch wounding 10A breast epithelial cells leads to **two Ca2+ signals:**
  1. Persistent edge
  2. **Neighboring wave**
- We have shown that the persistent edge signal is due to the XROS pathway (Pratt et al. *PNAS* 2020)
- The neighboring signal was thought to likely be caused by cells bursting or releasing ATP, which caused a wave to neighboring cells (Pratt et al. *Oncotarget* 2018)
- We have been able to inhibit the neighboring ATP-induced Ca2+ signal by inhibiting ATP with Apyrase and blocking the P2Y2 receptor
- Many cancer biology studies have shown that ATP concentrations vary in the tumor microenvironment.
- Rather than using complicated, time-consuming techniques, could we simply use ATP as a stimulus of wounding and what are the physiological outcomes of ATP messaging in breast tumors?

RNA Sequencing Data

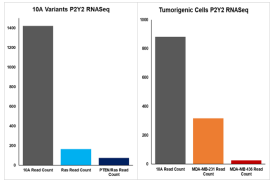


Figure 1. Our RNA Seq data showed that mRNA expression of P2Y2 receptor was down multiple folds in our mutant 10A-Ras and PTEN/Ras cell lines as well as metastatic MDA-MB-231 and MDA-MB-436 cell lines, when compared to non-tumorigenic 10A cells. 231's and 436's also harbor an activating KRas mutation.

P2Y2 Western Blot

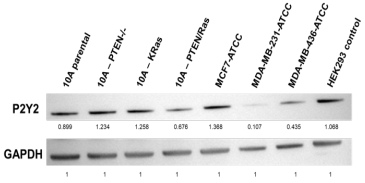
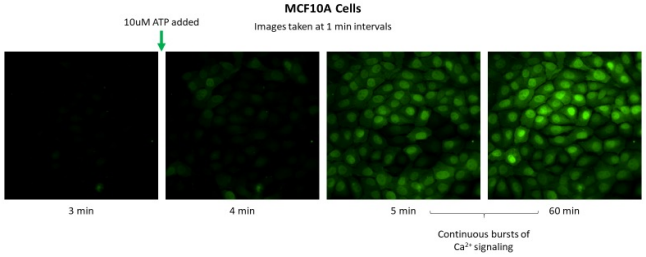


Figure 2. To further confirm our RNA sequencing results, we showed a decrease in P2Y2 receptor protein expression in various epithelial and tumorigenic cell lines. P2Y2 protein is down in our 10-PTEN/Ras mutant line, MDA-MB-231, and 436 lines. We also included a HEK293 positive control.

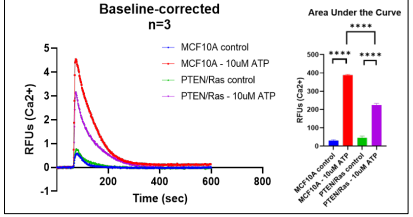
ATP Stimulates Ca^{2+} Flux

Figure 3. After previous data showed that ATP was the signal causing Ca2+ flux after wounding, we decided to move forward using ATP as an individual stimulus. Cells were loaded with Fluo-4 AM Ca2+ dye in HANKs Balanced Salt Solution buffer with Ca2+. Then we used fluorescent Nikon imaging to show that ATP can be used directly as a Ca2+ stimulus. 10uM ATP diluted in pH7 ddH2O was added around 3:30 mins directly into the buffer while imaging. Still images were taken from the compiled Nikon video at various time points to show changes in fluorescence. Controls were done in parallel, where we saw no significant changes in fluorescence.



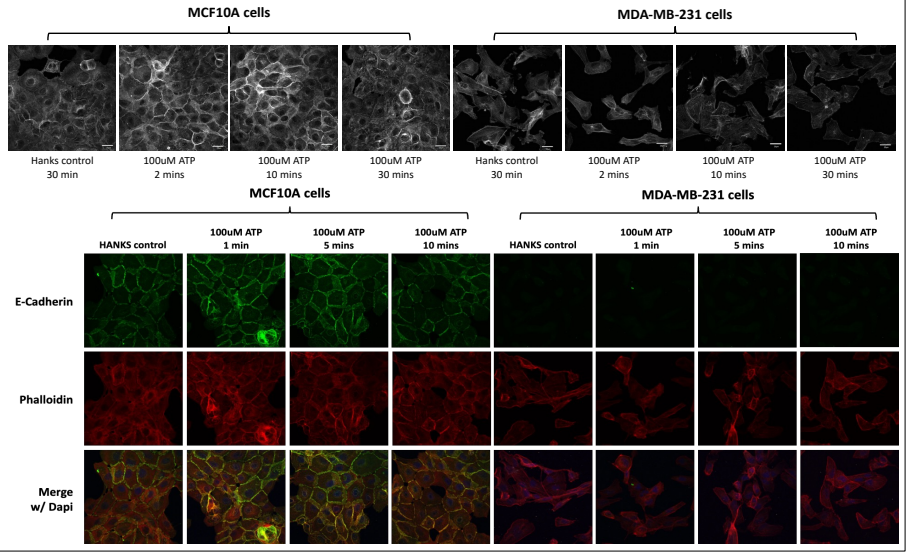
Quantitative Ca^{2+} Response with ATP

Figure 4. Further analysis of change in fluorescence was quantified using a FLEX plate reader. Cells were grown in 96-well imaging dishes and loaded with Fluo-4 AM dye in HANKs. We saw a significant difference in fluorescence between control and 10uM ATP treated cells, as well as differences in our normal 10A epithelial cells and the PTEN/Ras mutated cells. Area under the curve statistical analysis was performed in GraphPad Prism. N=3 and each experimental condition was a triplicate.



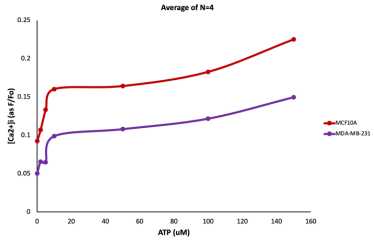
Changes in Actin and Cellular Junctions

Figure 6. Change in the actin cytoskeleton could be occurring in response to wounding or high levels of ATP. MCF10A cells and MDA-MB-231-ATCC cells were treated with 100uM ATP for varying time points and then fixed with 3.7% formaldehyde. Immunofluorescence staining was performed using phalloidin, and later E-cadherin. The normal 10A cells show a higher concentration of actin at cell-to-cell junctions when treated with ATP for short time periods, compared to no treatment or the metastatic 231 cells. E-cadherin is also localized to the cell-to-cell junctions and is more concentrated after ATP treatment in 10A cells, while 231 cells have no native E-cadherin. This further shows that 231 cells do not respond to normal Ca^{2+} signals.



Differences in intracellular Ca^{2+}

Figure 5. Similar to Figure 4., further comparison between normal breast epithelial MCF10A cells and metastatic breast cancer cells, MDA-MB-231-ATCC cells. Using the CLARIOstar Plus plate reader to quantify differences in intracellular Ca^{2+} concentrations. Cells were grown in 96-well plastic dishes and loaded with Fluo-4 AM dye in HANKs. Readouts were normalized to minimal and maximal fluorescence, which was found using EGTA and ionomycin in zero Ca^{2+} HANKs. We saw a difference in fluorescence between 10A cells and 231 cells. 10A cells seem to have a higher intracellular Ca^{2+} concentration and are stimulated at lower ATP concentrations compared to 231 cells. N=4 and each experimental condition was a quintuplet.



Conclusions

- ATP alone can induce a Ca2+ response in breast epithelial cells and breast tumor cells
- RNA sequencing and western blots show a decrease in P2Y2 receptor mRNA and protein in cell lines with KRas mutations
- Metastatic breast cancer cell line has a lower intracellular Ca^{2+} concentration compared to normal breast epithelial cells.
- Calcium fluxes induced by ATP can lead to numerous physiological phenotypes, like changes in actin and cellular junction proteins.

Future Directions

- Further studies on actomyosin changes
- IF with other actin components and cellular junctions
- What is causing the release of Ca2+ after P2Y2 activation?
- Will this act through G-proteins to stimulate IP3 receptor and release of intracellular Ca2+ stores?
- Does P2Y2 activation lead to another receptor activation on the plasma membrane to allow an extracellular flood of Ca2+ into the cells?
- Planning experiments with inhibitors for ATP, P2Y2, intracellular Ca2+ stores, and extracellular Ca2+ stores

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