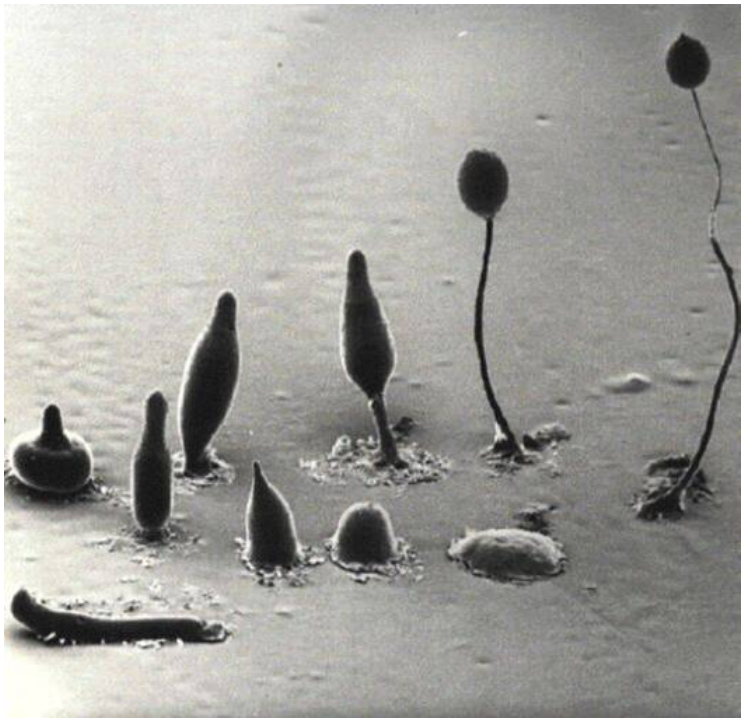
# The Effect of PKC Inhibitors and Activators on Cell Aggregation of *Dictyostelium discoideum*

Vanessa Hardy[1], Paola Giraldo[2], and Derrick Brazill[2]
[1]Edward R. Murrow High School Brooklyn, New York
[2]Hunter College/City University of New York, New York, New York



## Abstract

*Dictyostelium discoideum,* a single cell amoeba, is a model organism used to study signal transduction within a cell, as well as cell-cell communications. A low cell density assay was performed to test the hypothesis that Pkc inhibits PldB in the signal pathway responsible for cell aggregation. Two types of cells were used in the assay: wild-type cells (Ax2) and *pldB* gene knockout cells (β12). When cells are starved *pldB*$^-$ cells can aggregate at low cell density while wild type cells cannot. In the assay, starving cells were treated with Pkc inhibitors and activators. We predicted that wild type cells would aggregate at low cell densities when treated with Pkc activators and would not aggregate even at high cell densities. When treated with Pkc inhibitors *pldB*$^-$ cells were predicted to aggregate at low cell densities regardless of Pkc inhibitors and activators, for they lack *pldB* gene, thus the PldB protein. After various trials, Activator seem to slow down aggregation in Ax2 and β12 cells which is opposite of what was expected. Rapamycin which suppose to act like an inhibitor acts more like an activator.

## Introduction

Quorum sensing is known as cell density sensing in *D. discoideum* which is required for differentiation and development of *Dictyostelium* life cycle. Quorum sensing in *Dictyostelium discoideum* is accomplished by sensing the glycoprotein conditioned medium factor (CMF). Aggregation occurs in cells when the starving cells density is high and CMF levels are high. Quorum sensing can be seen in bacteria as well as eukaryotes like *D. discoideum* and mammals. *Dictyostelium discoideum* is used in lab research because of close similarity to human cells, enabling scientists to better study cell-cell communication and development. (Chen and others 2005).

*Dictyostelium discoideum* is a social amoeba that is most widely studied as a model organism for eukaryotic cell signaling, gene expression, and development. These bacteria-eating cells undergo various developmental stages and have extensive life cycles. *D. discoideum* have the ability to survive as a single-cell or multi-cellular organism. For example, it forms fruiting bodies when there is an absence of food. The structure of the *D. discoideum* fruiting body consists of a spore head supported by a long stalk with a round structure at the bottom that looks like a disk. In the spore head, cells are alive but dormant, but the stalk cells are dead. The development of these fruiting bodies takes about 24 hours. During the development of *Dictyostelium*, cell signaling, chemotaxis, and cellular differentiation occur. (Kendrew, 1994)

The mechanism used in *D. discoideum* is the cell densing sensing system. Scientists that are studying *D. discoideum* cell type mechanisms try to get cells to differentiate at low cell density. Mechanisms show that cell to cell contact is not necessary for differentiation (Gomer, Richard 1999). The conditioned medium factor

(CMF) is a secreted factor that is also necessary for differentiation in cells. CMF consists of two size fractions which have CMF activity, the larger fraction is an 80-kDa polypeptide which is secreted by starved cells and is used for cell density sensing during development. Many sequence data that were collected shows that CMF may be a new class of protein signal. (Gomer, Richard 1999).

During early aggregation, cells become sensitive to cAMP due to the expression of the cAMP receptors. The cells gain the ability to synthesize and secrete cAMP in response to cAMP stimulus. The secreted cAMP diffuses to activate other cells that in turn will start to produce cAMP and stimulate other cells. The cells show a chemotactic reaction in the direction of a higher cAMP concentration. As the cAMP concentration rises, the cells move up the gradient (Dormann and others 2000).

Chemotaxis plays an important role during the aggregation of *Dictyostelium* cells. Chemotaxis uses a signaling pathway that allows cells to sense or respond to chemoattractant gradient. Chemoattractants also cause aggregation. Cells can also sense a small concentration in differences in chemoattractant that lead to a change in direction and speed of cell movement. Changes in cell movement result from the activation of receptor-mediated signaling pathways at the edge of the cell, nearest the chemoattractant source. When the cells are exposed to the chemoattractant gradient, their movement follows the direction of the chemoattractant source. This chemotactic pathway is better understood in *Dictyostelium* cells than in mammalian cells. To form a multi-cellular organism up to $10^5$ cells, *Dictyostelium discoideum* chemotaxis to cAMP (Firtel and Chung 2000).

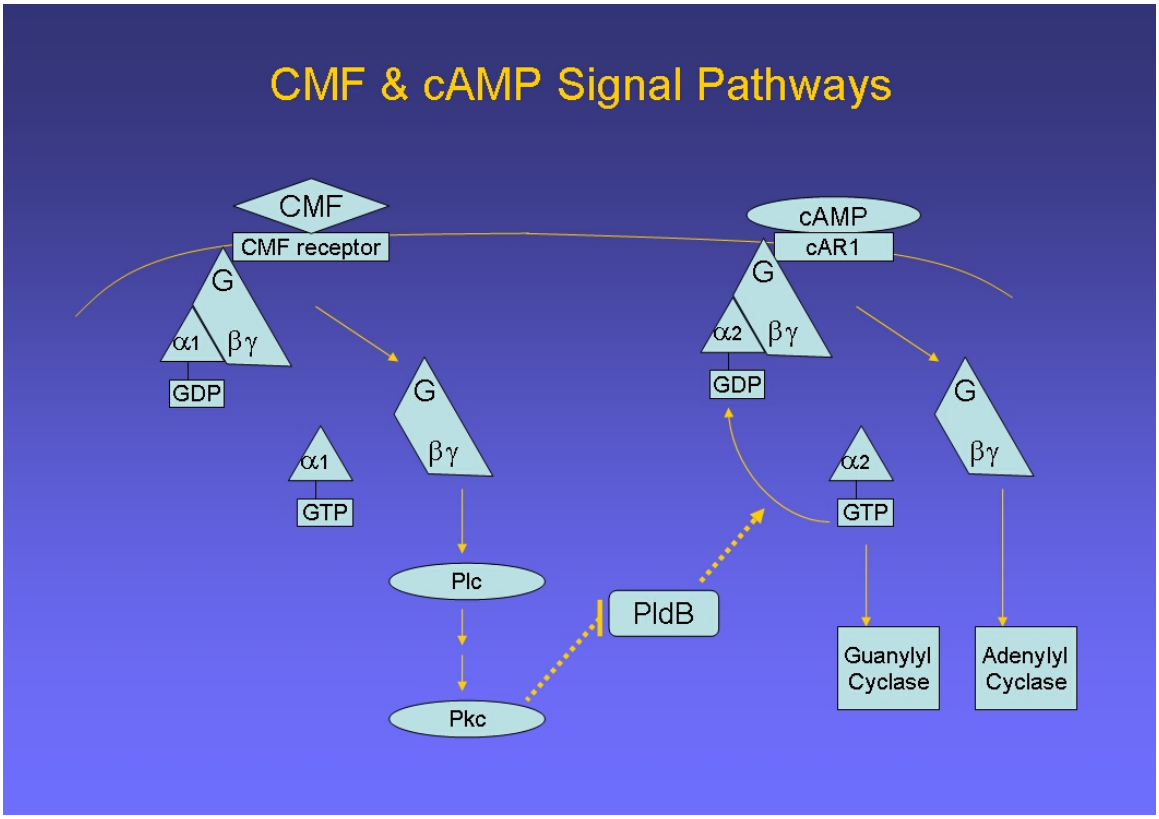
PldB, a putative phospholipase D homologue is known to be a negative regulator of quorum sensing. If the activity of PldB was removed, then the cells would aggregate at any density. If the activity of PldB is increased, the cells can't aggregate even at high cell density. In order to conduct the experiment, Chen and others (2005) starved wild type cells and PldB over expressing cells in a submerged monolayer culture to observe the effect of conditioned medium on cell aggregation. The wild type cells were able to aggregate when starved at low cell density with CMF. Cells that were over expressing PldB were unable to aggregate at high cell density, even in the presence of CMF. The experiment proved that PldB regulates activities in quorum sensing and is a negative regulator of quorum sensing. Data that were collected shows that PldB lies in the CMF pathway (Chen and others 2005).

In this experiment a particular section of the CMF and cAMP signal pathway (see Fig. 1) was studied, which involves the interaction of Pkc and PldB and its effect on cell aggregation. The purpose of this experiment is to determine whether Pkc does inhibit PldB as shown in the signal transduction pathway theorized through previous experiments.

It is believed that when cells are starved, and the cAMP receptor is activated, it causes activation of a signal-transduction pathway, which involves the separation of the G-protein's 2 subunits. The Gα2 subunit becomes GTP-bound and this result in activations required for motility/chemotaxis, while the second subunit, Gβγ leads to gene expression. The cell self-regulates by activating or deactivating the G-protein, when the Gα2 becomes GDP bound it is deactivated and the cells will no longer continue to aggregate. It is hypothesized that PldB may be a negative regulator of this pathway as

well by maintaining the Gα2 subunit inactive, therefore not allowing for cell aggregation. It is also hypothesized that through the CMF pathway, Pkc is activated and inhibits PldB activity in the cell, allowing the cells to aggregate.

**Figure 1. CMF and cAMP Signal Pathways**




## Material and Methods

To test the hypothesis that Pkc inhibits PldB in *Dictyostelium discoideum* signal transduction pathway, the experiment was designed using a low-cell density assay. Ax2 and β12 cells were treated with Pkc inhibitors and activators and their ability to aggregate at low cell density was monitored.

Using aseptic technique, cell concentration was first determined. In order to perform the assay cells need to be at a concentration between 2-5 x $10^6$cells/ml. Cell concentration was calculated by counting cells on a hemocytometer. To make sure that enough cells were placed in wells (approximately 6.8 x $10^5$ cells in 800µl for the first well), 10 times the amount of cells needed were collected: 6.8 x $10^6$ cells. The assay included two controls, Phosphate Buffer Media (PBM) solution and Dimethyl Sulfoxide (DMSO) the solvent used to dissolve treatments. The treatments were: (inhibitor 1) Bisindolylmaleimide diluted to a concentration of 50nM in PBM and DMSO; (inhibitor 2) Calphostin C at a concentration of 25nM; (activator 2) 1-Oleoyl-2-acetyl-*sn*-Glycerol at 75µM, and Rapamycin at a concentration of 1mM.

One 15ml falcon tube contained Ax2 cells and another 15 ml falcon tube contained *pldB*$^-$ cells. 6.8 x $10^6$cells were placed into the falcon tubes. The falcon tubes were then centrifuged at 1300 rpm for 5 minutes. After centrifugation, the medium was decanted without disturbing the cell pellet. The pellet was washed/resuspended with 10ml of treatment solution. The tubes were centrifuged again at 1300 rpm for 5 minutes. Cells were then resuspended in 8ml of treatment solution. Then 1ml of cells are placed in six Eppendorf tubes, and 1µl of DMSO, Inhibitor 1or 2, Activator 1 or 2, and 2µl of Rapamycin are added respectively.

Three 24-well plates were set up by adding 400µl of treatment solutions to columns 2-6 for all rows. 800µl was taken out of the Eppendorf tubes and loaded in the 1$^{st}$ well. Then 400µl from the 1$^{st}$ well were transferred into the 2$^{nd}$ well, and 400µl from the 2$^{nd}$ well into the 3$^{rd}$ well, which continued through the 6$^{th}$ well. Finally, 400µl were taken from the last well and discarded. This resulted in a 1:2 serial dilution, with each

well containing half the number of cells as the previous. The three 24-well plates were placed in a glass tray and left in a 21°C incubator for 18 hours. Then the cells were observed on an inverted microscope and results were recorded on a chart.

## Results

The hypothesis stated that Pkc inhibits PldB in *D. discoideum* resulting in aggregation of starved cells at low cell density. The results of the experiment did not reflect our hypothesis.

**TABLE 1**

| Cell +Treatment | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Ax2+PBM | +++ | ++ | ++ | - | - | - |
| β12+PBM | +++ | ++/ss | ++/ss | + | + | - |
| Ax2+DMSO | +++ | ++ | ++ | +(1) | - | - |
| β12+DMSO | ++/ss | sss | ++/ss | +/s | +(1) | +(1) |
| Ax2+INH 1 | +++ | ++ | ++ | - | - | - |
| β12+INH 1 | sss | ss | ss | + | + | - |
| Ax2+INH 2 | +++ | ++ | ++ | +(1) | - | - |
| β12+INH 2 | sss | ss | +/ss | +/ss | + | - |
| Ax2+ACT 2 | +++ | ++ | - | - | - | - |
| β12+ACT 2 | sss | ss | s | - | - | - |
| Ax2+Rapamycin | +++ | ++ | ++ | + | - | - |
| β12+Rapamycin | ++/ss | ss | ss | +/s | +/s | - |

| | Many | Some | Little | None |
|---|---|---|---|---|
| Aggregates: | +++ | ++ | + | - |
| Streaming: | sss | ss | s | |

**DEVELOPMENTAL STAGES**

**Figure 2. Aggregated cells**

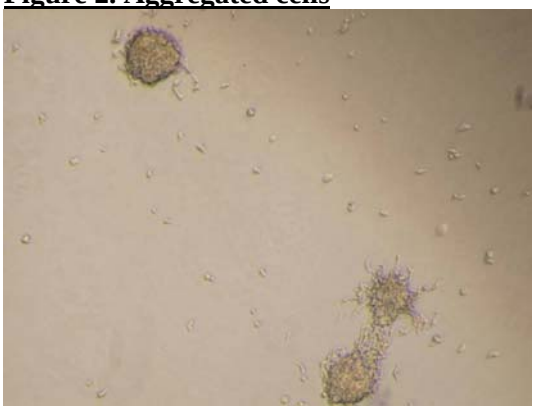


**Figure 3. Streaming cells**

**Figure 4. Initial Streaming Cells**

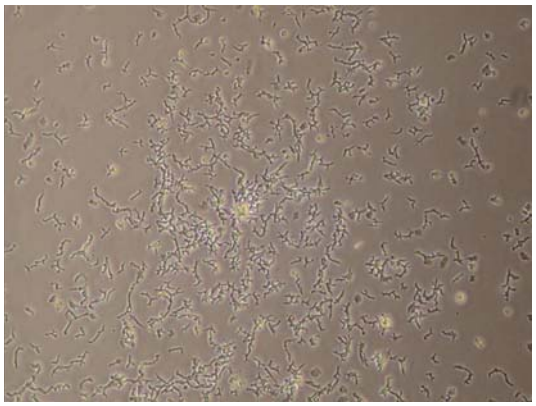


**Figure 5. No Aggregation: Single cells**

According to Table 1, Ax2 cells treated with PBM aggregated (see Fig. 2) up to the third well, and no aggregation (see Fig.5) in the fourth, fifth, and sixth well. Ax2 cells treated with DMSO showed aggregation from the first to third well, only one aggregate in the fourth well and no aggregation in the last two well. Ax2 treated with inhibitor 1 showed aggregation up to the third well and no aggregation for rest of the wells. Inhibitor 1 had no effect. Ax2 treated with inhibitor 2 showed the same results for Ax2 treated with DMSO. Inhibitor 2 also had no effect. Ax2 treated with activator 2 showed aggregation in the first and second well and no aggregation in the rest of the wells. This shows activator

2 partially blocks quorum sensing. Ax2 treated with Rapamycin showed aggregation from the first to the fourth well and no aggregation in the fifth or sixth well. This shows Rapamycin activates quorum sensing.

*PldB*$^-$ cells treated with PBM showed aggregation in the first well, aggregation and streaming (see Fig.3) in the second and third well, aggregation in the fourth and fifth well, and no aggregation in the last well. *PldB*$^-$cells treated with DMSO showed aggregation and streaming in the first well, streaming in the second well, aggregation and streaming in the third and fourth well, and one aggregate in fifth and sixth well. *PldB*$^-$ cells treated with inhibitor 1 showed streaming up to the third well, aggregation in the fourth and fifth well, and no aggregation in the last well. Inhibitor 1 had no effect. *PldB*$^-$ cells treated with inhibitor 2 showed streaming in the first and second well, aggregation and streaming in the third and fourth well, aggregation in the fifth well, and no aggregation in the last well. Inhibitor 2 also had no effect. *PldB*$^-$ cells treated with activator 2 showed streaming up to the third well and no aggregation from fourth to the last well. Activator 2 was partially blocking quorum sensing in the *PldB*$^-$ cells. *PldB*$^-$ cells treated with Rapamycin showed aggregation and steaming in the first well, streaming in the second and third well, aggregation and little streaming (see Fig. 4) in the fourth and fifth well, and no aggregation in the last well. Rapamycin had no effect on *PldB*$^-$ cells.

The data concludes that the cells used in this experiment were not normal. There was also some slight difference observed between the controls, PBM and DMSO. Rapamycin allows the cells to aggregate at low cell density which means it acts more like

an activator. The activator also acts more like an inhibitor because Ax2 and β12 cells aggregate up to the second or third well.

## Discussion

The results of this assay didn't support the hypothesis because the hypothesis stated that Pkc inhibits PldB in *D.discoideum* resulting in aggregation of starved cell at low cell density. If the hypothesis were true we would see Ax2 cells treated with Pkc Inhibitors would slow down the cells from aggregation at low cell density . Ax2 cells treated with Pkc Activator would speed up the cells to aggregate at low cell density. *PldB*$^-$ cells treated with Pkc Inhibitors and Activator would aggregate at low cell density. However, we saw Ax2 cells treated with activator 2 slows down the cells from aggregating at low cell density and Rapamycin seem to speed up the cells which suppose to act like a PldB inhibitor. The *PldB*$^-$ cells will aggregate and stream in the first well, some cells aggregate in the fourth and fifth well, and streaming also appeared in the first well down to the third well. *PldB*$^-$ cells treated with activator only shows streaming but no aggregation. *PldB*$^-$ cells treated with Rapamycin showed aggregation and streaming from the first well down to the fifth well.

The expected results were that when Pkc inhibitors were added to Ax2 cells it would lead to no aggregation; Pkc activators added to Ax2 cells would lead to aggregation at low cell density; β12 cells treated with Pkc inhibitors would result in aggregation at low cell density; and Pkc activators added to β12 would result in aggregation at low cell density. Once PldB is blocked aggregation will occur at low cell density.

A possible source of error that could have happened in this experiment is that both Ax2 and β12 cells were not used at an ideal developmental concentration (2-5 $x10^6$cells/ml) during the experiment. If the cells were used at a higher or lower concentration than the ideal for the experiment, the cells would not develop correct. Another possibility in this experiment is that the concentration of the activators and inhibitors were used at a low concentration resulting in no effect due to treatment.

## Conclusion and Future Research

In conclusion, the hypothesis which stated that Pkc blocks PldB in *D. discoideum* results in aggregation of starved cell at low cell density was not supported by all the data that was gathered in the experiment. The results showed that Activator seem to slow down aggregation in Ax2 and β12 cells which is opposite of what was expected. Rapamycin which suppose to act like a PldB inhibitor acts more like an activator.

In addition more research is needed to establish if Ax2 overexpressor cells (overexpressing the pldB gene) will result in aggregation at low cell density. Future research is also needed to test different concentration of inhibitors and activators in *D. discoideum* cells to determine the concentration needed to observe differences.

## References


Chen Yi, Rodrick V, Yan Yi, Brazill D. 2005. PldB, a putative phospholipase D homologue in *Dictyostelium discoideum* mediates quorum sensing during development. Am Soc for Microbiol 4:694-702.

Dormann D, Vasiev B, Weijer C. 2000. The control of chemotactic cell movement during Dictyostelium morphogenesis. Phil Trans R Soc Lond B 355:983-991.

Firtel R, Chung C. 2000 The molecular genetics of chemotaxis: sensing and responding to Chemoattractant gradients. Bio Essays 22:603-615

Gomer R. 1999. Cell Density Sensing in a Eukaryote. ASM News 65: 23-29

Kendrew J. 1994.The Encyclopedia of Molecular Biology. Blackwell Science Ltd 262-64

## Acknowledgements

I would like to thank Harlem Children Society and Project Seed for giving me a great opportunity to work in a lab with undergrad and graduate student at Hunter College. I also want to thank my mentor Dr.Brazill for giving me the opportunity to work in his lab by experiencing and learning new thing each day. I would like to give a special thank you to my other mentor Paola Giraldo for guiding me, teaching me new things in the lab, giving me good advice and for helping me organize my paper. I would like to give another thank you to Yi Chen for the pictures that are in this paper and helping me organize my paper.