The Role of Extracellular ATP in *Dictyostelium discoideum* Growth and Development

Peter F. Woolman

A Thesis Presented to
School of Biological Sciences, University of East Anglia

In Fulfilment of the Requirements of the University of East Anglia for the Degree of
MSci By Research

December 2013

© This copy of thesis has been supplied on the condition that anyone who consults it is understood to recognise that its copyright rests with the author and that no quotation from the thesis, nor any information derived from it, may be published without the author's prior written consent.

Declaration

I hereby declare that the work in this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Signature: ……………………………………….

Date: …………………………………………….

Abstract

It has long been known that the social amoeba *Dictyostelium discoideum* initiates and controls its developmental processes by the creation and transmission of the purinergic molecule; cyclic AMP. It is also known that it possesses P2X receptors which are localised on the inside of the cell. Despite the lack of cell surface ATP receptors, previous studies have indicated the existence of *Dictyostelium* ecto-ATPases and calcium responses to the addition of exogenous ATP.

In these experiments it was found, using the luciferase assay, that the cells condition their media with ATP during axenic growth and development. Treatment of cells with apyrase, a potato enzyme known to degrade ATP, did not affect the rate of growth, but led to a delay in the appearance of streams during development. This delay corresponded with the formation of "clumps" of cells, but no evidence could be found that the cells had increased expression of adhesion molecules.

Apyrase also caused developing cells to form fewer fruiting bodies, despite the fact that there was no difference in the number of aggregates formed, implying that the apyrase was interfering in the transition between these stages. The apyrase fruiting bodies also contained fewer viable cells, though whether it was fewer cells in general or less healthy ones could not be confirmed.

The malachite green assay was used to measure the rate of activity of the cell surface ecto-ATPases and the results suggested that the common ecto-ATPase inhibitor suramin would affect them. Cells allowed to develop in the presence of suramin showed no abnormal timing in the stages of development or in number of fruiting bodies, but did contain significantly more viable cells.

Altogether these results are consistent with the hypothesis that ATP, ATP receptors and ATPases play a role in the regulation and co-ordination of development in *Dictyostelium discoideum,* despite the fact that it possesses no known cell surface P2X receptors.

Table Of Contents

Abstract 3

Table Of Contents 4

List Of Figures 8

List Of Abbreviations 12

Acknowledgements 13

Chapter 1 - Introduction 14

1.1 – Purinergic Signalling 14

1.1.1 - Purinergic Signalling - History 14

1.1.2 – Purinergic Signalling – Mechanisms Of ATP Export From Cells 14

1.1.3 – Purinergic Signalling – Mechanisms Of Extracellular ATP Detection 15

1.1.4 – Purinergic Signalling – ATP Breakdown 16

1.2 - *Dictyostelium discoideum* 17

1.2.1 – Dictyostelium discoideum - Background 17

1.2.2 – Dictyostelium discoideum – Development 18

1.3 – There Are Potentially Further Purinergic Signalling Systems Regulating Development In *Dictyostelium discoideum* 20

Chapter 2 - Materials And Methods 22

2.1 - Cell Culture 22

2.2 - Use Of The Haemocytomer 22

2.2- Measuring Growth Curves 23

2.3 - Development Buffer 24

2.4 – Development In Submerged Culture 24

2.4.1 - Treatment With Apyrase 24

2.4.2 - Treatment With Heat-Inactivated Apyrase 24

2.7 – Development On Agar 25

2.8 - Determining Fruiting Body Size 25

2.9 – Measuring Extracellular ATP 26

2.10.0 – Measuring Cell Adhesion 26

2.10.1 - Aggregation Quotient 26

2.10.1 – Measuring Adhesion 26

2.11.0 – Measuring ATPase Activity 27

2.11.1 – Phosphate Liberation Buffer 27

2.11.2 – MLG Reagent 27

2.11.3 – Molybdate Solution 27

2.11.4 – Measuring Phosphate Liberation 27

2.12 – Data Analysis 27

Chapter 3 – Investigating The Presence Of Extracellular ATP And The Effect Of Its Removal Upon Growth And Development In *Dictyostelium discoideum* 29

3.1 - Aim 29

3.2 – Introduction 29

3.3 –*Dictyostelium discoideum* Undergoing Exponential Growth Causes Luciferase To Produce More Luminescence Than Untreated Buffer 30

3.4 –The Addition Of Exogenous Apyrase Has No Effect On Exponential Growth Of *Dictyostelium discoideum* 31

3.5 - *Dictyostelium discoideum* Secretes ATP During Development 32

3.6 - Submerged Culture 33

3.6.1 - Submerged Culture - Characterising The Control 33

3.6.2 - Submerged Culture – The Addition Of Exogenous Apyrase To Dictyostelium Cells Causes A Developmental Delay 36

3.6.3 - Submerged Culture – The Addition Of Exogenous Apyrase Impacts The Development Of Dictyostelium Cells That Are Already Undergoing Development. 40

3.6.4 - Submerged Culture – Heat Inactivated Apyrase Is Incapable Of Causing The Apyrase Induced Delay 40

3.6.5 - Submerged Culture – The Addition of Extracellular Adenosine Deaminase To Dictyostelium Does Not Counteract the Apyrase Induced Delay In Stream Formation. 41

3.6.6 – Submerged Culture – The Effect Of Exogenous Apyrase On Dictyostelium Development Is Not Limited To The AX4 Strain 43

3.7.0 – Cell Adhesion 45

3.7.1 Cell Adhesion – Characterising The Control 45

3.7.2 - Cell Adhesion –Addition Of Exogenous Apyrase To Dictyostelium Does Not Alter the Aggregation Quotient 47

3.8.0 – Development On Agar 50

3.8.1 – Development On Agar – The Addition Of Exogenous Apyrase Induces A Developmental Delay In Dictyostelium discoideum *Developing On Agar* 50

3.8.2 - Development On Agar – The Addition of Exogenous Apyrase Decreases the Number of Fruiting Bodies formed by Dictyostelium discoideum 51

3.8.3 - Development On Agar – The Addition Of Exogenous Apyrase Decreases the Number Of Viable Cells Within Fruiting Bodies Produced By Dictyostelium discoideum 51

3.8.4 – Development On Agar – Investigating Whether There Is A Link Between Number Of Fruiting Bodies And Number Of cells Within The Fruiting Body Heads 53

3.9 – Conclusions 53

Chapter 4 – Investigating Ecto-ATPases And Their Role In Regulating Development In *Dictyostelium discoideum* 58
4.1 – Aim 58
4.2 - Introduction 58
4.3 – Phosphate Liberation 59
4.3.1 – Phosphate Liberation – Measuring Change In Absorbance In Control Conditions 59
4.3.2 – Phosphate Liberation – Measuring Change Of Absorbance In The Presence of Suramin 61
4.3.3 – ATPase Activity – Measuring Change Of Absorbance In The Absence Of Mg^{2+} Ions 62
4.4 – The Effect Of Suramin On *Dictyostelium* Development In Submerged Culture 63
4.5 – The Effect Of Suramin On *Dictyostelium* Development On Agar 66
4.6 - Conclusions 68
Chapter 5 - Bioinformatics 71
5.1 – Aim 71
5.2 – Introduction 71
5.3 – Overview Of The Process Used 72
5.4 – Conserved Sequences 73
5.5 – Protein BLAST Search 78
5.6 – Transmembrane Alignment 79
5.7 – Identifying Domains 80
5.8 – Results 80
5.8.1 – Results - DDB_G0276135 80
5.8.2 – Results – Other genes 82
5.9 – Conclusions 83
Chapter 6 - Discussion 85
6.1 – An Overview Of The Findings Of This Project 85
6.2 –*Dictyostelium discoideum* Responds To Increased Degradation Of Extracellular ATP 85
6.3 – Addition Of Apyrase To *Dictyostelium* Causes A Delay In The Initiation Of Streaming And The Formation Of Clumps 87
6.4 – Alterations In The Rate Of Breakdown Of Extracellular ATP Causes A Change In The Number Of Viable Cells Making Up *Dictyostelium discoideum* Fruiting Bodies 89
6.4.1 – The Rate Of ATP Degradation May Affect The Number Of Cells Making Up A Dictyostelium discoideum Fruiting Body 89
6.4.2 – The Rate Of ATP Degradation May Affect The Viability Of Cells Making Up A Dictyostelium discoideum Fruiting Body 90
6.5 – Addition Of Apyrase To *Dictyostelium discoideum* Causes A Decrease In The Number Of Fruiting Bodies Formed. 91

6.6 – What Further Work Could Be Done To Answer The Questions Raised By This Project?...... 91

References...... 93

Appendix - Dictyostelium Genes Identified By The BLAST Analysis That Had Been Determined To Contain Two Transmembrane Domains And The Correct Topological Alignment...... 99

List Of Figures

Figure 1 – Use Of The Haemocytometer. a) The view of the counting grid of a haemocytometer loaded with *Dictyostelium discoideum* cells b) the same haemocytometer with the squares to be counted labelled 23
Figure 2 – Increased Luminescence From The Luciferase Reaction In Culture Medium Conditioned by Growing *Dictyostelium*. The luciferase-luciferin reaction results in significantly less luminescence in unconditioned FM minimal growth media than media conditioned with growing *Dictyostelium* ($5x10^5$ cells/ml; 17 hours at 125RPM). The error bars represent standard error of the mean of each data point, N=4, P<0.05. 30
Figure 3 – Growth Curves Of *Dictyostelium.* Growth over a period of 54 hours in FM minimal media under control conditions or in the presence of 4U/ml apyrase. The error bars represent standard error of the mean of each data point, N=4, P>0.05. 31
Figure 4 - Detection Of ATP Upon Addition Of *Dictyostelium* To Development Buffer. Mean ATP detected by luciferase-luciferin reaction (luminescence, RLU) over the 120 minutes following seeding of *Dictyostelium* into development buffer at $5x10^5$ cells/ml. Each data point is subtracted from the value obtained at the same time point from buffer that did not contain cells. The error bars represent standard error of the mean of each data point. 32
Figure 5 –Development In Submerged Culture. *Dictyostelium* development in submerged culture at cell density of $1x10^6$cells/ml. Photos taken at 4, 7, and 24 hours after addition to the wells. Images are annotated with descriptions of the developmental process. 33
Figure 6 - Developmental Progress Of *Dictyostelium* After 14 Hours At 4 Different Cell Densities (cells/ml). Aggregates of cells are visible at densities of $1x10^6$cells/ml and $5x10^5$cells/ml. 34
Figure 7 – Development Of *Dictyostelium* In Submerged Culture At Cell Density Of $5x10^5$cells/ml. Between 14-16 hours cells begin to elongate and lose their amoeboid shape. At 18 hours the elongate cells begin to sort themselves into patterns and very early streams, only a couple of cells long, can be seen. By 20 hours the streaming is occurring, the cells are forming a vortex shape as they determine where the eventual aggregation centres will be. These aggregation centres are visible at 22 hours and the cells are moving inwards along the streams towards them. By 24 hours the streams have begun to break up, each segment will form their own separate aggregate. 35
Figure 8 – Apyrase Causes A Delay In The Formation Of Streams. Development of AX4 cells at a density of $5x10^5$cells/ml in cultures treated with 4U/ml of apyrase or an equivalent volume of water over a period of 24 hours. Time since addition to the buffer is given in hours on the left of the image. Photos are of the most developmentally advanced cells found within the well. In control wells streams are first visible at 20 hours and begin to break up by 24 hours. The Apyrase-treated cells, however, take a further two hours before they start to form streams. 37
Figure 9 – No Effect Of Apyrase On Complete Aggregate Formation. After 30 hours in development buffer, aggregates of control *Dictyostelium* cells at a density of $5x10^5$cells/ml do not have observable differences to those formed in the presence of 4U/ml apyrase 38
Figure 10 – Apyrase Causes The Formation Of "Clumps". After treatment with 4U/ml apyrase, *Dictyostelium* at a density of $5x10^5$cells/ml form "Clumps." Images taken 1.5 hours after additon of cells to the buffer 38
Figure 11 - Evidence For Increased Incidence Of Cells Failing To Commit To Aggregate Formation Upon Treatment With 4U/Ml Apyrase. Cells at $5x10^5$cells/ml. Images taken at 20hours after addition to the buffer. 39
Figure 12 –Apyrase Induced Developmental Delays Caused No Significant Difference In The Eventual Number Of Aggregates Formed. Number of aggregates present in wells containing control

and apyrase (4U/ml) treated cells following completion of development at two different densities of *Dictyostelium.* The error bars represent standard error of the mean of each data point, N=7. Each data point is expressed as a percentage of the average amount of aggregates within each pair's control well. 39
Figure 13 – Streaming Is Halted By Addition Of Apyrase. Apyrase (4U/ml) was added to *Dictyostelium* cells at a density of $5x10^5$cell/ml half an hour after the starting of stream formation. Time in hours since addition of cells to the buffer is recorded in the top left corner of each image. ... 40
Figure 14 – No Observed Effect Of Heat-Inactivated Apyrase On Development. Cells after 24 hours at $5x10^5$cells/ml under control conditions or with apyrase (4U/ml) that was either enzymatically active or heat inactivated 41
Figure 15 – Adenosine Deaminase Has No Effect On The Timing Of *Dictyostelium* Stream Formation. Photo taken 13 hours after addition to development buffer. In the wells that do not contain apyrase the cells are forming streams. In the wells that do contain apyrase there are no streams present but cells are forming clumps. Cells at a density of $5x10^5$ cells/ml under control conditions or with apyrase (4U/ml) and with or without adenosine deaminase (2U/ml)........ 42
Figure 16 – Additive Effect Of Apyrase And Adenosine Deaminase On Number Of Aggregates Formed During Development. Number of aggregates present in wells seeded with $5x10^5$cells/ml *Dictyostelium* under control conditions or treated with apyrase (4U/ml), adenosine deaminase (2U/ml) or both proteins after a period of 42 hours. The error bars represent standard error of the mean of each data point, N=3 with two wells in each experiment........ 43
Figure 17 - Apyrase Causes A Delay In The Initiation Of Streaming Of AX2 Cells. Development of AX2 cells seeded at a density of $5x10^5$cells/ml in cultures treated with 4U/ml of apyrase or an equivalent volume of water over a period of 24 hours. Time since seeding is given in hours on the left of the image. Photos are of the most developmentally advanced cells found within the well........ 44
Figure 18 – Aggregation Quotient Changes Over Time Under Control Conditions. *Dictyostelium* cells at a density of $5x10^5$cells/ml adhere to each other when mixed in the buffer through chance collisions and the expression of adhesion molecules a) Mean Aggregation Quotient over a period of 4.5 hours. The error bars represent standard error of the mean of each data point, N=8. b) Representative figure of the change of aggregation quotient in one tube over a period of 4.5 hours........ 46
Figure 19 –Addition Of Apyrase Has No Effect Upon The Aggregation Quotient. The change in Aggregation Quotient over time of *Dictyostelium* at a density of $5x10^5$cells/ml is not altered when apyrase (4U/ml) is added. The Aggregation Quotient is, however, altered by addition of EDTA (5mM) which prevents cell-cell adhesion. Control N=15, apyrase N=9, EDTA N=6. The error bars represent standard error of the mean of each data point. 47
Figure 20 - EDTA Prevents Development. *Dictyostelium* seeded at $5x10^5$ cells in submerged culture over a period of 20 hours either with or without exogenous EDTA (5mM). Control cells start to form streams at 15 hours and by 20 hours are beginning to become complete aggregates. EDTA treated cells fail to show any signs of development. 48
Figure 21 – Rounded Appearance of EDTA Treated Cells. Within 1.5 hours of being added to the submerged culture, *Dictyostelium* seeded at $5x10^5$cells/ml and treated with EDTA (5mM) are noticeably rounder than ones in control conditions 49
Figure 22 – Apyrase Causes Delayed Development On Solid Agar. Development of AX4 cells seeded at a density of $1x10^4$ cells/cm^2 on solid non nutrient agar. The cells treated with 4U/ml water are still at the mound and slug stages of development after 22 hours. At this time the control cells are mostly at the fruiting body stage with some remaining slugs........ 50
Figure 23 – Apyrase Causes A Decrease In The Number Of Fruiting Bodies Formed. Mean number of fruiting bodies formed by *Dictyostelium* seeded at a density of $1x10^4$ cells/cm^2 on solid non nutrient

agar with or without apyrase (4U/well). Each data point is expressed as a percentage of the relevant control. The error bar represent standard error of the mean the data, N=6, two wells/experiment. 51
Figure 24 - Evidence A For Smaller Number Of Viable Cells In Fruiting Bodies Developed During Apyrase Treatment. Number of colonies produced by 0.5ml of the mixture of a fruiting body head and 4ml of *Klebsiella*. Spores were produced from by *Dictyostelium* seeded at a density of $1x10^4$ cells/cm^2 on solid non nutrient agar under control conditions or in the presence of 4U/well of apyrase. Each data point is expressed as a percentage of the relevant control. The error bars represent standard error of the mean the data, N=24 plates of control and 28 of apyrase. 52
Figure 25 - The Number Of Fruiting Bodies In A Well Containing $2.5x10^6$ Cells Plotted Against The Mean Number Of Colonies Produced By Said Fruiting Bodies. 53
Figure 26 – There Is Significant Change In The Absorption Of light From Samples Taken From Wells Containing Cells After Addition Of Exogenous ATP Between Time Zero And Either Three Minutes Or Six Minutes. Data at three minutes is significantly different in each of the samples from wells containing cells from the control wells at the same time point ($p<0.05$). There is no statistical difference at six minutes between the wells containing cells and the control that do not, but the wells containing cells still have significantly more absorbance ($p<0.05$) than the same well at time zero. Data points taken at 3 and 6 minutes. The error bars represent standard error of the mean of each data point, N=7 60
Figure 27 – In The Presence Of Exogenous Suramin, Exogenous ATP Causes No Alteration In Absorbance At 600nm In The Six Minutes After Addition. Addition of ATP (0.5mM) in the presence of suramin (250µM) causes no significant difference in absorbance of light at a wavelength of 600nm. Data points taken at 3 and 6 minutes. The error bars represent standard error of the mean of each data point, N=6 61
Figure 28 – Removal Of Mg^{2+} Ions From The Reaction Buffer Decreases The Rate Of Ecto-ATPase Activity. Addition of ATP (0.5mM) with MgCl left out of the reaction buffer causes a significant difference in absorbance of light at a wavelength of 600nm between samples taken from the wells containing cells at time zero and the same wells at six minutes. No significant difference was observed in the absorbance of light between samples taken from wells at time zero and three minutes in any of the wells. Data points taken at 0,3 and 6 minutes. The error bars represent standard error of the mean of each data point, N=6 62
Figure 29 – No Effect Of Suramin On Development. Development of AX4 cells at a density of $5x10^5$cells/ml in cultures treated with 250µM of suramin or an equivalent volume of water over a period of 24 hours. Cells start to form early streams at 5.5 hours and by 6.5 hours streaming is fully underway. By 24 hours aggregates have formed. Time is given in hours on the left of the image. Images are of the most developmentally advanced cells found within the well. 64
Figure 30 – Treatment With Suramin Caused No Significant Difference In The Number Of Aggregates Formed By *Dictyostelium*. Number of aggregates present in wells containing control or suramin (250µM) treated *Dictyostelium* seeded at $5X10^5$cells/ml following completion of development. The error bar represents standard error of the mean of the data, N=3, 2 wells/experiment 65
Figure 31 – Suramin Has No Effect On The Timing Of *Dictyostelium Discoideum* Development On Agar. Development of AX4 cells seeded at a density of $1x10^4$ cells/cm2 on solid non nutrient agar treated with 250µM of suramin or an equivalent volume of molecular-grade water at 19 and 43 hours after addition to the plate. At 19 hours cells in all the wells had formed mounds. By 43 hours all the plates contained fruiting bodies. 66
Figure 32 - Suramin Has No Effect On The Number Of Fruiting Bodies Formed. Mean number of fruiting bodies formed by *Dictyostelium* seeded at a density of $1x10^4$ cells/cm^2 on solid non nutrient

agar with or without suramin (250µM). Each data point is expressed as a percentage of the number of fruiting bodies in the paired control well. The error bar represents standard error of the mean the data set, N=7, two wells/experiment. 67
Figure 33 - Evidence For Increased Number Of Viable Cells In Fruiting Bodies Developed In The Presence Of Suramin. Number of colonies produced by 0.5ml of the mixture of a fruiting body head and 4ml of *Klebsiella* when grown upon an agar plates. Spores produced from *Dictyostelium* seeded at a density of $1x10^4$ cells/cm^2 on solid non nutrient agar with or without suramin (250µM). Each data point is expressed as a percentage of the number of colonies produced by the relevant control. N=29 plates of control and 21 of suramin. The error bar represents standard error of the mean the data set. 67
Figure 34 - Sections Of The Clustalw Output (Larkin et al., 2007) With The Regions That Were Highly Conserved Marked And Numbered 77
Figure 35 –TMHMM Output For Four Proteins (Analysis, 2007). a) Protein gi_66826167 has four transmembrane domains, which is too many so it is discarded. b) Protein gi_66822343 has no transmembrane domains. It cannot be anchored in the membrane and so it is discarded. c) Protein gi_66822343 contains two transmembrane regions, but the segment between them is located on the inside of the cell membrane. Even if this protein has ATPase activity, it would be acting on nucleotides on the inside of the cell and so is irrelevant to this investigation. It is discarded. d) Protein gi_66810896 has two transmembrane domains separated by an external region. This is carried on for further investigation. 79
Figure 36 - TMHMM Output For Gene DDB_G0276135 (Analysis, 2007) 81

List Of Abbreviations

This list provides clarification to some of the abbreviations that are commonly used throughout this thesis

ADP – Adenosine DiPhosphate

AMP – Adenosine MonoPhosphate

ATP – Adenosine TriPhosphate

cadA – Gene that expresses csB protein

csB – Contact Site B adhesion molecule

cAMP – Cyclic Adenosine MonoPhosphate

EDTA - Ethylenediaminetetraacetic Acid

EGF – Epidermal Growth Factor

EGTA - Ethylene Glycol Tetraacetic acid

E-NTPDase - Ecto-Nucleoside TriPhosphate Diphosphohydrolase

HL5 – A media for axenic *Dictyostelium discoideum* growth composed of yeast

FM Minimal – A media for axenic *Dictyostelium discoideum* growth containing no yeast

MLG – Malachite Green

P1 – A class of Adenosine detecting Purinergic Receptor

P2X – A class of Purinergic ATP Receptor

P2Y – A class of Purinergic Receptor to ATP and ADP

PDE – Phosphodiesterase

PSF – Prestarvation Factor

UTP - Uridine TriPhosphate

Acknowledgements

First and foremost thanks must go to Dr Samuel Fountain, who gave me the opportunity to work in his laboratory, carry out this project and always had helpful recommendations. I also thank the other members of the Fountain Lab: Dr Venketesh Sivaramakrishnan, who introduced me to the tenets of *Dictyostelium* culture and development, and Hinnah Campwala, who kept me company in the lab and provided feedback on my work at weekly lab meetings. I am also deeply indebted to my secondary supervisor, Professor Tracey Chapman, for helping me obtain this project and for providing advice and guidance whenever I lost track of the bigger picture.

I would like to thank my parents who raised me, encouraged me to pursue a career in research and provided financial support during this project. Thanks also go to the students of the Pamela Salter Room, who guided me in the ways of Post-Graduate life, and Florence Morgan-Richards, who provided essential emotional support.

Finally I thank my Grandfather, Dr John Peter Whitehouse, who regaled me with tales of scientific curiosities in my youth and who inspired me to become a scientist.

Chapter 1 - Introduction

1.1 – Purinergic Signalling

1.1.1 - Purinergic Signalling - History

Traditionally neurotransmission has been thought of as being divided into the two categories cholinergic and adrenergic, with nerves releasing either noradrenaline or acetylcholine. It was not until the 1970s that the case was put forward for the existence of a third category, though, in retrospect hints as to its existence were seen as early as the late nineteenth century. This third category was tenatively called purinergic because, at the time, it was believed to use only a single purine nucleotide as the signal (Burnstock, 1972). Since these early investigations purinergic ATP signalling has been found in almost all nerves in both the central and peripheral nervous systems of mammals, while purines and pyramidines carry signals in virtually all human non-neuronal tissues (Burnstock and Verkhratsky, 2009).

The ubiquitous role of ATP as the primary energy source of cells has given rise to the belief that it was selected for this task very early on in evolution and that, as a result, even the most primitive early cells would have had a high internal ATP concentration. When these cells died their degradation would have released this ATP, causing ATP gradients in the primordial seas. It is believed that this is what initially led to the development of the purinergic signalling systems. Cells evolved mechanisms to detect this gradient in order to avoid dangerous places where cell death was common (Burnstock and Verkhratsky, 2009). Over time ATP receptors have grown more elaborate with more specialised purposes.

Its early appearance in evolution explain why ATP seems to be the main exception to the rule that extracellular signalling molecules are functionally and anatomically segregated. ATP is not only found in specific tissues doing specific tasks. Today it can be found in a wide variety of roles and tissues (Burnstock and Verkhratsky, 2009) including, but not limited to; acting as a neurotransmitter in smooth muscle (Mutafova-Yambolieva, 2012), modulating Ca^{2+} signalling in too many different contexts to list here (Mammano, 2012), modulating the immune system in ways which are exploited by cancers (Pellegatti et al., 2008) and even maintaining homeostasis in bones (Ham and Evans, 2012). ATP also posseses a wide variety of release pathways.

The widespread nature of ATP signalling implies that it appeared very early in evolution (Burnstock and Verkhratsky, 2009) and it is the wide variety of its functions which makes ATP-regulated purinergic signalling such an important field of study. Interestingly, while the ATP-sensitive P2X receptors are found in a wide range of organisms, from humans to amoeba, they are also absent in many organisms such as higher plants, fruit-flies and yeasts (Sivaramakrishnan and Fountain, 2012). The phylogeny of other ATP-sensing receptors, such as the P2Y receptor is less clear with reports concerning their expression in plants and simple organisms, including Dictyostelium, lacking.

1.1.2 – Purinergic Signalling – Mechanisms Of ATP Export From Cells

There are several ways in which extracellular ATP can be released from the cell, the first of which has been alluded to already. If a cell can be induced to burst, such as in the event of osmotic cytolysis, it will cause the release of all its contents: including the 3-5mM ATP found within the cytoplasm under

normal, healthy circumstances (Beigi et al., 1999). This is a non-specific mechanism of ATP release. Extracellular ATP is released along with the rest of the cytoplasmic contents and, of course, it results in the death of the cell from which the ATP originates. This would rarely be a deliberate action by the cell, but it would provide an unintended warning signal to other cells in the vicinity. This is probably the initial mechanism from which the entire purinergic network has evolved (Burnstock and Verkhratsky, 2009).

A second way in which ATP can be released from the cell is as part of a secretory vesicle. Proteins synthesised within the cell, but which need to be localised to the outside of the cell membrane, or even into the extracellular space, are packaged into membrane-enclosed containers called "vesicles." These are then transported to and fuse with the cell's membrane, releasing the vesicle's contents on the outside of the cell (Sherwood, 2012). It is through this method that more traditional signalling molecules, such as noradrenaline in nerves, are released from the cell. It is now known that ATP can also be packaged inside these vesicles for release (Lohman et al., 2012).

ATP Binding Cassette, or ABC, transporters are a type of membrane protein which move molecules across the plasma membrane which might otherwise not be able to do so due either to their polarity or diffusion gradients. The energy for this process is gained by the removal of a phosphate from ATP and thus each of these proteins possess two conserved ATP binding domains (Borst and Elferink, 2002). There is, however, a growing school of thought that suggests some of these transporters not only use ATP as an energy source for transport, but actually transport it as well. Such an idea is still controversial. As of the time of writing papers are still regularly being published with conflicting evidence on the subject. Some scientists even suggest that the ABC transporters do not release ATP themselves, but regulate an unknown ATP release mechanism (Lohman et al., 2012). The existence of ABC transporter-mediated ATP release is not confirmed. The conflicting evidence, however, means that it must be considered as a potential source of extracellular ATP in studies where it can be found to be present without a known release pathway.

Traditionally ATP has been considered as being created inside cells. In mammals the ATP synthase protein has traditionally been believed to exist only within the mitochondria. In another example of advances in the field of purinergics overturning established knowledge of ATP, however, there is evidence to suggest that, in some forms of human tumour cells at least, ATP synthase homologous molecules exist on the outside of the cell membrane (Das et al., 1994). This suggests there are some cells capable of generating extracellular ATP from ADP directly in the extracellular environment without needing to export it.

1.1.3 – Purinergic Signalling – Mechanisms Of Extracellular ATP Detection

There are nineteen types of purinergic receptors which are sorted into three classes: P1 receptors which respond to adenosine and the two forms of nucleotide triggered P2 receptors; G protein-coupled P2Y and ionotropic P2X (Mathieu, 2012). The P2X receptors are then further divided into P2X1-P2X7 subfamilies (Surprenant and North, 2009), while the P2Y receptors have eight subfamilies. Unlike the neat 1-7 nomenculature of the P2X receptors, the P2Y families consist of P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14. The numeric gaps represent proteins that, while homologous to P2Y receptors, have not been shown to have any response to nucleotides. It was also only after the classes had been subdivided in this way that it was discovered that some P2Y receptors respond to pyrimidines instead of purines (Abbracchio et al., 2006).

P2X receptors are gated channels, which respond to ATP with a rapid change in permeability to allow Na^+, K^+ or Ca^{2+} cations to pass through. P2X7 is unique amongst them, in being the only one whose pore is capable of acting non-selectively (Ralevic and Burnstock, 1998).

A P2X subunit consists of two transmembrane domains, named TM1 and TM2 (Jiang et al., 2012). The bulk of the protein consists of an extracellular hydrophilic loop of amino acids, which separates them. It is important to note that a subunit alone is incapable of forming an ion channel (Ralevic and Burnstock, 1998). One P2X receptor is believed to be composed of three subunits with each pore being made up of three separate TM2 domains surrounded by a ring of three TM1 domains (Jiang et al., 2012). Unlike the P2Y receptors (except P2Y11), P2X receptors contain introns in their coding sequence (Abbracchio et al., 2006).

Sequence analysis of P2X receptors from various species reveals that the regions that are highly conserved, and which are essential for the function of the protein in mammals, are found mostly in the transmembrane domains (Surprenant and North, 2009). The vast bulk of the ecto-domain, which had previously been considered to be the crucial part, is barely conserved at all. There are four amino acids in the area immediately following the transmembrane domain: Tyr^{55}, Gln^{56}, Lys^{69} and Lys^{71}. These are the only amino acids known to be vital for receptor activation. It is now believed that only the Lys residues are essential for ATP binding (Surprenant and North, 2009). Crystalography of the Zebra Fish P2X receptor suggests that, in this specific receptor at least, the binding site resembles an open jaw. When ATP binds to this, the jaw “tightens” and this conformational change in the protein shape opens up the pore (Jiang et al., 2012).

P2Y receptors are rhodopsin like g-coupled proteins consisting of seven transmembrane domains (von Kügelgen and Wetter, 2000). These respond as much as ten times slower than P2X receptors to extracellular ATP (Ralevic and Burnstock, 1998). Each subfamily of P2Y receptor can couple to a wide range of different g-proteins in order to give a wide variety of different effects. It has been suggested that this variety of different g-proteins can itself give rise to specificity to which agonist the receptor binds. For instance P2Y11 receptors exposed to ATP will increase cAMP and Ca^{2+} levels. When P2Y11 receptors are exposed to UTP, however, only Ca^{2+} will be released, while the cAMP permeable receptors remain unactivated (Abbracchio et al., 2006).

1.1.4 – Purinergic Signalling – ATP Breakdown

Another important component of purinergic signalling systems are the ATPases which hydrolyse ATP. These are essential in that, not only do they terminate the signal activating the P2X receptors, but they also generate ADP, which activates the P2Y receptors. Furthermore, many ATPases are also capable of breaking down this ADP further into AMP (Knowles, 2011). In mammals CD73 proteins then convert this AMP into adenosine, which activates P1 receptors (Colgan et al., 2013). In this way one molecule of ATP can briefly activate many receptors as it is broken down (Knowles, 2011).

The class of ATPases responsible for ATP hydrolysis in purinergic signalling is the ecto-ATPase. This consists of membrane-bound proteins, which are insensitive to the main inhibitors of most other classes (Chadwick and Frischauf, 1997) and which require Ca^{2+} or Mg^{2+} ions to function (Kukulski et al., 2005). It was initially thought that only one ecto-ATPase existed, named CD39, with a variety of post-translational modifications. As more were discovered, however, the existence of the entire ENTPDase (ecto-nucleoside triphosphate diphosphohydrolase) family was acknowledged (Robson et al., 2006).

All of the eight known ENTPDase proteins, except 5 and 6, are located on membranes with 1,2,3 and 8 being on the cell's external membrane. They are connected to the membrane by two transmembrane helices with the catayltic region on the outside of the cell in order to act upon the extracellular ATP. These transmembrane domains, and indeed their presence within the cell membrane, are critical for the catalytic ability, at least in the CD39 protein, as they hold the active site in specific shapes. It is believed that the variety of active site shapes caused by transmembrane anchoring is responsible for the variety of substrate specificities present in the ENTPDase family (Grinthal and Guidotti, 2006).

1.2 - *Dictyostelium discoideum*

1.2.1 – Dictyostelium discoideum - *Background*

Dictyostelium discoidium is a model organism that is used in a wide variety of fields from studies of phagocytosis, to cell signalling, cell motility (Montagnes et al., 2012), cell adhesion and even evolution (Abedin and King, 2010). In 1998 an initiative to sequence the *Dictyostelium discoideum* genome was launched; the first attempt to sequence the full genome of a free living protozoan. The completed genome was published in *Nature* in 2005 (Eichinger et al., 2005), thus further increasing its popularity and usefulness as a model organism. *Dictyostelium* is of particular use in this study because it has been known since 2007 to contain P2X receptors (Fountain et al., 2007) and was thus confirmed to use ATP as a signalling molecule. Later research indicates that the known receptors are all positioned to detect ATP on the inside of the cell membrane and thus this signalling is all intracellular (Ludlow et al., 2009). Ludlow et al (2008), however, demonstrated that extracellular ATP could activate a hitherto unknown calcium influx pathway, suggesting that there is expression of cell surface ATP receptors in *Dictyostelium*.

Cellular slime moulds are used in developmental studies due to the fact that they are amongst the simplest eukaryotic organisms capable of acting multicellularly (Bracco et al., 2000). Although this multicellularity is known to have evolved independently of the same phenotype in animals and plants, many of the same gene families were vital for both transitions (MacWilliams et al., 2006). Furthermore, while this multicellularity is achieved not through growth and differentiation of cells, but through aggregation (Dormann et al., 2000), it retains many of the same developmental pathways of the more difficult to study metozoans from which they diverged millenia ago (Coates and Harwood, 2001).

During the early stages of their life cycle slime moulds, such as *Dictyostelium discoidium*, typically exist as solitary amoeba, reproducing via binary fission and feeding on bacteria found within their environment by phagocytosis (Bracco et al., 2000). Axenic strains of many have been created in the laboratory to ease the undertaking of genetic studies (Franke and Kessin, 1977).

Upon starvation many amoeba in an area come together to form an aggregate. The size of this aggregate can vary from 100 to 10000 cells (Bracco et al., 2000). This aggregate differentiates into stalk and spore cells. The stalk cells form a hard structure that hold aloft the spore cells, which have formed a sorus, thus allowing them to disperse over a large area. The spore cells are picked up by the wind and transferred to a new location where there is likely to be more nutrients available than their previous resource depleted area. Here they can continue their unicellular life cycle, while the stalk cells remain at the old location and die (Kuzdzal-Fick et al., 2010).

The altruistic nature of the life cycle of these cells seems counterintuitive from an evolutionary perspective. Dead cells do not get to reproduce. This is, in fact, a classic example of kin-selection

theory which states that cells (or higher organisms) will sacrifice themselves to ensure the reproduction of others, which are closely related and thus have a high chance of possessing and passing on the same genes (Velicer, 2005). In soil samples of *Dictyostelium* relatedness is 0.52, meaning that individuals on average share 52% of the same genes. The complex network of protein interactions which allows development to occur, however, also encourages fruiting bodies to comprise cells that are even more closely related. This significantly raises the average relatedness of cells within the same fruiting body (Gilbert et al., 2007). One interesting side effect is that if two different cultures of *Dictyostelium* are mixed prior to development, one of the cultures will preferentially make spore cells while the other will make stalk cells, even though normal distribution of stalk and spore cells occurs when they develop independently (MacWilliams et al., 2006).

1.2.2 – Dictyostelium discoideum – *Development*

Most of the timing of key events in the *Dictyostelium discoideum* developmental process is externally mediated as opposed to regulation by internal timers (Jain et al., 1992). It has been known for some time that purinergic signals play a vital role in this mediation. Although a wide range of chemoattractants are used to regulate development in slime moulds, the group 4 species (which include *Dictyostelium discoideum*) use cyclic AMP (cAMP) (Schaap, 2011).

The emission of this cAMP is triggered by alterations in the ratio between the concentration of the constantly emitted autocrine Prestarvation Factor (PSF) and the concentration of prey bacteria. PSF levels are themselves proportional to the cell density and in general PSF/bacteria ratios reach the critical point three to four generations before the bacterial levels become too low to support further exponential *Dictyostelium* growth (Burdine and Clarke, 1995).

Dictyostelium discoideum posseses four cAMP receptors known as cAR1-4, each with different affinities to the agonistic cAMP. For the initiation of development cAR1 and cAR3 are the most important with double mutants failing to chemotax or aggregate properly (Manahan et al., 2004). cAR1 is coupled to the g-grotein Gα2βγ on the inside of the membrane. Upon cAMP binding, the Gα2 subunit separates from the bulk of the protein (Ray et al., 2011), triggering the activation of adenyl cyclase (ACA) which generates further cAMP. This cAMP is then exported outside the cell where it is able to spread through the medium thus propogating the signal (Das et al., 2011).

Simultaneously, the gene *pdsA* encodes a phosphodiesterase (PDE) to degrade the extracellular cAMP and ensure that later releases of the signal cause a detectable change in concentration (Bader et al., 2006). Another effect of cAMP binding is to trigger phosphorylation of the cAR1 receptor, lowering its affinity to cAMP until it is rectified. This causes the receptors' responses to occur in bursts upon exposure as opposed to a constant release (Das et al., 2011). Bursts of cAMP, closely followed by cAMP degrading phosphodiesterases, result in the formation of the cAMP waves for which *Dictyostelium* is famous. Two splicing variants of the phosphodiesterase exist: a secreted one and a membrane bound one. Mathematical simulations suggest that the reason for these two forms is to provide optimal activity at different cell densities (Palsson, 2009).

This expression of both adenyl cyclase and phosphodiesterase results in the creation of waves of cAMP spreading throughout the medium from multiple aggregation centres. Like all waves, when these meet they experience both constructive and destructive interference: areas of high and low cAMP concentration (Palsson, 2009).

The cells are capable of detecting differences in concentration across their length, by determining how many of the Gα2 receptors are activated on each side (Kortholt et al., 2011). The cells are capable of

detecting very small differences in cAMP concentrations because the cells themselves are tiny compared to the wavelengths of the cAMP waves. They then chemotactically move in the direction of the higher cAMP concentration. This movement can only occur when the cAMP waves are passing over the cells and the cAR1 receptors are active. The result is periodic inward movement, which can be visualised using a timelapse microscope (Dormann et al., 2000). The movement of cells emitting cAMP changes the location of the high and low concentration areas, but the eventual result is the formations of clusters of up to $1x10^5$ cells around aggregation centres.

Careful observation of cells reveals that when the waves are passing over them and they are undergoing chemotaxis, they also undergo morphological changes. They became elongated and this changes their brightness when observed under a dark field microscope, allowing one to visualise the shape and progress of the cAMP waves (Dormann et al., 2000).

As the elongated cells converge upon the aggregation centre they connect head-to-tail forming, what are known as, chemotactic streams that extend the entire length of the aggregation territory. These streams help guide the cells efficiently towards the centre (Veltman and van Haastert, 2008) and by continuing to emit cAMP they greatly increase their range. When the cells enter the streams they start differentiating into the prespore and prestalk cells, which will eventually further differentiate into the spore and stalk cells. There is no correlation between their tendency to form one type or the other and their position within these streams (Weijer, 2009). At the aggregation centre, where the streams meet, they form a hemispherical mass known as the mound (Siegert and Weijer, 1995).

Cells within the streams are known to adhere to each other and this is mediated by multiple proteins, normally classifed by their resistance or lack of resistance to dissociation by EDTA. There is some evidence to suggest that EDTA-resistant proteins maintain end-to-end cell contact, whereas the EDTA-sensitive proteins are involved in mediating contact between side-to-side cells (Brar and Siu, 1993).

Gp24 is one such adhesion protein, which also goes by the names of DdCAD-1 and contact site B (csB) (Coates and Harwood, 2001). It is encoded by the gene *cadA*, which starts being expressed upon starvation in *Dictyostelium* grown on bacterial cell lawns. Expression of the mRNAs peaks, and starts to fall again, three hours before protein levels peak, but this peak level stays near constant due to the protein having a suprisingly large half life (Yang et al., 1997). Curiously *cadA* is also expressed during vegetative axenic growth at high cell densities, being induced by the PSF levels (Brar and Siu, 1993).

Over the course of aggregation the cytoplasmic gp24 is relocated to the plasma membrane (Sesaki and Siu, 1996). Gp24 is EDTA sensitive, which means that it is Ca^{2+} dependent. It contains two Ca^{2+} binding domains (Sesaki and Siu, 1996). Experiments in which non-membrane-bound gp24 is added to cells show a large reduction in cell adhesion, implying that the adhesion is created by homophilic interaction with the same protein on the membrane of neighbouring cells. There is only one binding site per protein. In these experiments only small aggregates of between 3-10 cells formed and over half of the cells present failed to develop (Brar and Siu, 1993). Blocking of gp24s binding sites does not prevent the formation of aggregates, but they are far looser and less compact (Siu et al., 1988).

The initial cAMP pulses also trigger expression of receptors, allowing the detection of DIF-1, which is needed to form two of the four subtypes of prestalk cells. Differentiation into prespore cells also requires extracellular cAMP, but there are several other compounds that are essential for it to occur (Yamada et al., 2010).

The mound forms a moving "slug" shape. The tip of the slug acts as a pacemaker, continuing to secrete cAMP periodically. The waves flow back along its length showing the later cells the direction in which to move (Jaiswal et al., 2012a). The slug moves to a point of high ground following light and temperature gradients (Flegel et al., 2011). As it moves the cells are sorted so that the front is composed mostly of the prestalk cells, while the posterior contains the prespore cells (Yamada et al., 2010).

Once the slug has reached a suitable place to form a fruiting body it stops moving, settling on its posterior and forming the so-called "mexican hat" stage. The tip begins to secrete a cellulose tube and the cells begin to ascend. The prestalk cells which were the closest to the tube initially end up around the base, while the further away prespore cells have further to climb to find a space and end up at the top (Tyler, 2003).

1.3 – There Are Potentially Further Purinergic Signalling Systems Regulating Development In *Dictyostelium discoideum*

cAMP is not the only purinergic molecule known to have an effect on the development of *Dictyostelium.* Adenosine is known to promote the formation of larger aggregates as well as inhibiting the synthesis of the cAMP degrading phosphodiesterases (Jaiswal et al., 2012b).

In 2007 bioinformatic techniques were used to detect five *Dictyostelium discoideum* genes that were very weakly homologous to human P2X receptors. When cloned in human HEK cells, these genes were confirmed to indeed be ATP receptors (Fountain et al., 2007). At the time it was believed that these receptors could be involved in cell-cell communication. The proteins encoded by these genes named *p2xA*, *p2xB*, *p2xC*, *p2xD* and *p2xE*, however, were found to all be located on the inside of the cell. They are now believed to be attached to internal vacuoles and control the release of Ca^{2+} ions from acidic stores (Sivaramakrishnan and Fountain, 2012).

The P2X receptors were initially believed to be involved in cell-cell communication because prior to their discovery there was already some evidence to suggest that exogenous ATP is capable of speeding up the onset of aggregation in *Dictyostelium discoideum.* Furthermore, the presence of ATP is known to cause an increase in the number of phosphorylated cAMP receptors earlier in development (Mato and Konijn, 1975). As well as this direct evidence for the role of extracellular ATP, it has been known for some time that *Dictyostelium discoideum* possesses cell surface ATPases (Parish and Weibel, 1980). It seems unlikely that *Dictyostelium discoideum* would possess proteins that have no use and this can be construed as secondary evidence for an extracellular ATP signal for these ATPases to terminate.

Following the discovery of the intracellular location of the P2X receptors, experiments were carried out to confirm that these earlier findings had been correct. Attempts were also made to explain these results using advances in the field of molecular biology made since the original observations. It was found that upon exposure to ATP, cells would react with a large and rapid increase in intracellular Ca^{2+} levels. At the same time it was discovered that ADP was capable of producing an almost identical calcium response while other nucleotides could not. The fact that the ATP response could be desensitized by exposure to ADP implied that both nucleotides were detected by the same receptor. This response was, however, inhibited by the addition of the P2X inhibitor gadolinium, implying a similiarity between the internal P2X receptors and whatever unknown receptor was giving this response. Further evidence that this response was initiated by a P2X receptor was provided by mutants which lacked the g-proteins required by P2Y receptors but still produced the calcium

response. It was, however, conclusively shown that the discovered P2X receptors were not involved in this response as mutants lacking these receptors still give it (Ludlow et al., 2009). The mechanism allowing *Dictyostelium discoideum* to undergo a P2X-like response without using P2X receptors is unknown.

The evidence is mounting that *Dictyostelium discoideum* uses extracellular ATP for an unknown purpose. In this project an investigation was launched into firstly discovering the circumstances under which extracellular ATP was released, and secondly the effect of removal of this extracellular ATP. Attempts were then made to discover the mechanisms responsible for this effect and from this deduce the role of extracellular ATP under control conditions.

Chapter 2 - Materials And Methods

2.1 - Cell Culture

A LB agar plate was completely spread with *Klebsiella* to act as a food source for *Dictyostelium discoideum*. Frozen stocks of the amoeba containing $1x10^7$ cells were then thawed on ice. Individual drops of this solution were then added to five evenly spaced areas of the plate. This plate was left in the $22^{O}C$ incubator to allow the *Dictyostelium* to grow and spread across the whole surface.

The rest of the thawed stock of cells were added to a petri dish already containing 10ml of HL5 growth media, 100U/ml penicillin and 100µg/ml streptomycin. The following day, when there were plenty of cells adhering to the bottom of the plate, the liquid from this media was added to a new plate and fresh media, penicillin and streptomycin added to the original plate. When spores had formed on the bacterial lawn plate, they were carefully removed and suspended in another plate also containing 10ml of HL5 media and 100U/ml penicillin and 100µg/ml streptomycin. The first of these three plates to produce a large culture of healthy looking cells (determined by microscopy) was then used for experiments.

The soup was removed from the healthy cells and discarded to eliminate any lingering bacterial contaminants. The cells adhering to the plate were washed and poured into a conical flask containing 20ml of HL5 media along with 100U/ml penicillin and 100µg/ml streptomycin. A sponge bung was placed on the flask to prevent contaminants from entering, while allowing fresh O_2 to enter and CO_2 to leave. This flask was then left on a shaking incubator set to 150RPM at room temperature. After 6 hours the cell density was calculated with a haemocytometer and, if necessary, more media was added to the flask to adjust the cell density to $1x10^6$cells/ml. Once the culture was confirmed to consist of healthy cells, aliquots of $1x10^7$cells were taken and frozen in order to provide future cultures.

Every 24 hours the cell density was calculated and cells were spun down in a centrifuge at 1500RPM for five minutes. The soup was removed and the cells were resuspended in a fresh 50ml flask of media with 100U/ml penicillin and 100µg/ml streptomycin. The number of cells spun down was calculated to be such that the cell density would be $1x10^6$cells/ml at the point when cells would be taken for experiments. Cells at a high density start to express developmental genes (Jain et al., 1992). Care was taken to ensure that cell density never exceeded $4x10^6$cells/ml as this was found to be the density at which this effect began to occur in these lab conditions. The 100U/ml penicillin and 100µg/ml streptomycin were added to the cell cultures to reduce the risk of them being infected with bacterial pathogens.

2.2 - Use Of The Haemocytomer

Before each use the haemocytometer was carefully cleaned and dried, then a clean coverslip was placed on top. 10µl of the sample was taken up with a pipette. The tip was carefully pressed against the side of the coverslip and the solution released, with care being taking to ensure the coverslip was not moved. For these experiments a two chambered haemocytometer was used, allowing two cell counts to be made before it needed to be washed and reloaded. Each side of the haemocytometer was loaded and then observed under a microscope. The grid upon the haemocytometer was located as in Figure 1a.

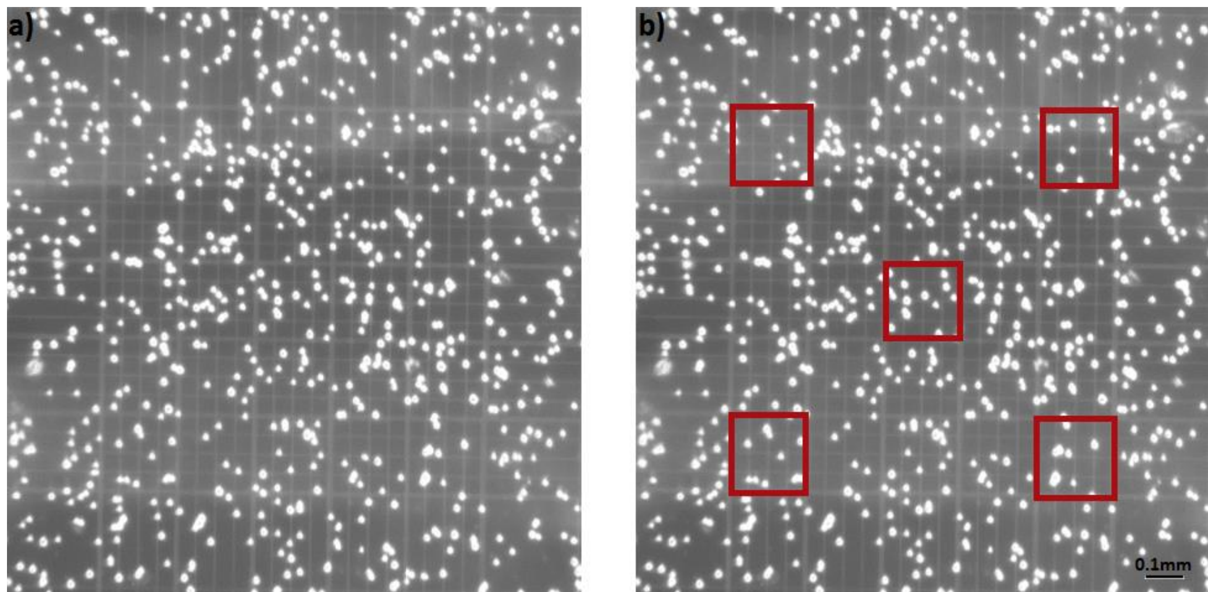


Figure 1 – Use Of The Haemocytometer. a) The view of the counting grid of a haemocytometer loaded with *Dictyostelium discoideum* cells b) the same haemocytometer with the squares to be counted labelled

The number of cells inside the four squares in the corners and the centre square were counted (Figure 1b). The top and left hand side of the squares were included while the other two sides were not. Each chamber was counted and then the haemocytometer was washed and reloaded so as to give a total of 4 counts. A mean of these counts was taken and then multiplied by 50,000 in order to give the number of cells found in 1ml of the sample.

2.2- Measuring Growth Curves

Two 10ml conical flasks were each filled with 3ml of cells at $5x10^5$cells/ml. To one of the flasks was added 60µl of 200U/ml apyrase to give a concentration of 4U/ml. To the other flask was added 60µl of Molecular Grade water to act as a control. The number of cells in each flask was calculated at 10:00am and 16:00pm every day using the haemocytometer. For these experiments one monitored the growth of the same culture of cells in the same media and flask throughout. The cells cultured normally in the lab for use in experiments were transferred to fresh media and flasks daily to prevent the build-up of pathogens and to ensure they always had fresh nutrients in the media. This could not occur in this experiment as culturing would impact the growth curves and wash out the apyrase. Each experiment was therefore continued until the cultures in the flask were visibly unhealthy. This experiment was repeated four times.

2.3 - Development Buffer

In many of the studies carried out, the cells were incubated in an isotonic development buffer. This consisted of Na_2HPO_4 (5mM); KH_2PO_4 (5mM); NH_4Cl (18.75mM); $MgSO_4$ (328µM); $CaCl_2$ (252µM).

This was adjusted to be pH6.8. This was then passed through a 50mm diameter filter with a pore size of 0.2µm in a UV sterilised hood into an autoclaved bottle. This bottle of buffer was kept at $0^{O}C$ until half an hour before an experiment, whereupon it was brought back to room temperature using a water bath.

In early preparations of the buffer it was autoclaved instead of filtered, but this led to the formation of a cloudy precipitate, which interfered with development and when viewed under a microscope was visually similar enough to bacterial contamination to cause confusion in the interpretation of results.

2.4 – Development In Submerged Culture

Two and a half hours before the experiment began, the cells were cultured using the methods described to a density of $5x10^5$ cell/ml. This was because pre-developmental cell density and the associated build up of PSF levels could influence the expression of certain developmental genes (Brar and Siu, 1993). By culturing the cells to the same density into fresh media a constant time before the experiments were due to start, one could attempt to minimise the effect of these factors and give consistency to repeated experiments.

At the start of the experiment the cell density was again calculated and the required volume of cells centrifuged for four minutes at 1800RPM and then resuspended in 20ml of development buffer. This was pelleted again under the same conditions and the buffer discarded twice more. The cells were then resuspended in enough buffer to give a relevant volume of cells at an appropriate density for the particular experiment. The multiple centrifugations were to help remove any contaminants in the original cultures, as well as to wash off any trace volumes of the HL5 media as this contains ATP (Min et al., 2006), which could interfere with these experiments.

A 24 well plate was filled with 1ml of the cell-buffer solution in each well the experiment required. The plate was then placed into an illuminated incubator set to $23^{o}C$. Over time the cells settled to the bottom of the well and adhered to the base. Cells were regularly observed under a microscope and quickly returned to the incubator. Photos were taken of the cells under the microscope if relevant.

2.4.1 - Treatment With Apyrase

Apyrase was prepared at a stock concentration of 200U/ml in molecular grade water. This was then sterilised by passing the solution through a 0.2µm filter.

The submerged culture experiments were set up as outlined in section 2.4. Half of the wells had 4U/ml apyrase added to them. These wells were labelled. The other half of the wells had an equal volume of molecular grade water added to act as a control. The *Dictyostelium* development within these wells was observed to see if the apyrase-treated cells displayed a phenotypic difference.

2.4.2 - Treatment With Heat-Inactivated Apyrase

Initial experiments were carried out in which apyrase was heat-inactivated at the stock concentration. This caused the protein to precipitate out of the solution as a sticky lump in which cells would get

trapped. In an attempt to counter this effect, for the heat-inactivation experiments, the apyrase stock was added to Eppendorf tubes of development buffer at a concentration of 8U/ml. These were placed in a 95°C heatblock for ten minutes before being removed and allowed to cool slowly to room temperature.

Wells were prepared as in section 2.1, but with the wells containing 0.5ml of cells at twice the density. 0.5ml of the heat-inactivated apyrase in solution was added to give a final density of both cells and apyrase as found in the previous experiments.

For the sake of maintaining control conditions throughout the experiments, the well containg apyrase-treated cells were also prepared during these experiments at 8U/ml in development buffer with 0.5ml added to 0.5ml of cells at 1x10^{6}cells/ml. The same was done for the control cells, but with unchanged development buffer added in place of apyrase solutions.

2.7 – Development On Agar

1.5% mass per volume bacteriological agar was dissolved in development buffer. A microwave oven was used to heat the buffer sufficiently for the agar to dissolve. 3ml of this agar solution was added to each well of a 6 well plate and allowed to solidify for 1-3 hours. During this process the lid was placed back on the wells to stop them drying out.

2.5x10^{6} cells were taken for each well used in the experiment and resuspended in development buffer at 1x10^{7}cells/ml using the same washing procedure as in the submerged culture experiments. 0.2ml of this solution was then transferred to an Eppendorf for each well. If required, 4U of apyrase in 20µl in molecular grade water were added to each of these Eppendorfs. The contents of the Eppendorf were then mixed slightly with a pipette and added to the centre of one of the agar filled wells from earlier. A plastic spreader was used to ensure even distribution within the well.

The plate was wrapped in damp paper to prevent the agar drying out, and placed in the incubator upside down so that evaporated moisture would condense near the cells. Development typically occurred over 2-4 days. The plates were observed and redampened frequently over this time.

2.8 - Determining Fruiting Body Size

Single fruiting body heads were extracted with a damp pipette tip and resuspended in 4ml of *Klebsiella*. 0.5ml of this solution was added to two agar plates and spread evenly with separate spreaders. These were left in an incubator until colonies developed. These colonies were counted and the mean number of colonies produced from each head calculated. Initially the heads were suspended in 1ml of *Klebsiella* which was completely spread on the plates, but with so much *Dictyostelium* on a plate, the limiting factor for growth became not the number of viable cells, but amount of space on the plate so a more suitable dilution was used.

2.9 – Measuring Extracellular ATP

ATP release was measured in submerged culture and exponential growth. In these experiments the cells were set up as in the relevant experiment above together with a control sample of just the relevant media or buffer. At each timepoint 50μl of the solution was taken from both the sample and the control and added to individual fresh Eppendorf tubes. These were then centrifuged at -4°C for 4 minutes at 2300RPM in order to separate the cells and their contents from any secreted products. The low temperature was used in order to limit natural ATP degradation.

Once the centrifugation was complete, 25μl of the supernatant was removed from the Eppendorfs and placed into a fresh Eppendorf. 25μl of 0.1mg/ml luciferase was added to each Eppendorf and they were placed in a biophotometer in order to measure light emitted with an integration time of 7 seconds.

2.10.0 – Measuring Cell Adhesion

2.10.1 - Aggregation Quotient

Aggregation was calculated according to an "Aggregation Quotient" derived by comparing the number of lone cells under the haemocytometer to the number of groups of cells. This was used instead of comparing the number of lone cells to the number of cells adhering to each other because it was hard to accurately determine the number of cells making up the larger groups. The Aggregation Quotient was calculated as follows:

$$Aggregation\ Quotient = \frac{Number\ of\ Groups\ of\ Cells}{Number\ of\ Groups\ of\ Cells + \ Number\ of\ Lone\ Cells}$$

It is important to note that the Aggregation Quotient is the ratio of aggregates to lone cells, as opposed to a measure of how many cells have aggregated. An Aggregation Quotient of 1 would mean that all the cells present had become part of aggregates and one of 0 would mean that none had. A quotient of 0.5 would mean that there were an equal number of aggregates as lone cells as opposed to 50% of the total cells being aggregated, which one might expect it to mean in other systems.

The Aggregation Quotient at time zero was never found to be zero. This was because some cells were in the process of splitting into two via binary fission. It is not believed that an increase in Aggregation Quotient could be caused by this binary fission process because this only occurs during the axenic growth phase and cannot occur in the buffer-induced starvation conditions (Saxer et al., 2010).

2.10.1 – Measuring Adhesion

A relevant volume of cells were suspended at 5x10^{5} cells/ml in development buffer. 1.5ml of this solution was then added to 2ml centrifuge tubes. Chemicals such as EDTA and apyrase were added at this stage as required. These tubes were attached to a vertical rotating wheel in such a way that each half turn would invert the tubes. The 0.5ml air pocket was to ensure that the contents would be adequately mixed. This device was set to 46 RPM. Every 30 minutes the device was stopped and 10μl of the tubes' contents loaded onto a haemocytometer slide. The number of groups of cells and the number of lone cells were counted and the Aggregation Quotient calculated. Three tubes of each treatment were set up in each experiment, so as to minimise variation between tubes.

2.11.0 – Measuring ATPase Activity

2.11.1 – Phosphate Liberation Buffer

Phosphate liberation buffer consists of Tris-maleate (0.1M); $MgCl_2$ (2mM); KCl (2mM); pH 7.2 with KOH.

The pH of the buffer was adjusted to 7.2 by careful addition of KOH. This buffer precipitates if left at room temperature for a couple of days, so it was made up either on the day of the experiment or the night before and kept on ice until needed.

2.11.2 – MLG Reagent

MLG Reagent consists of malachite green (1.0mM); polyvinyl alcohol (0.16% w/v); H_2SO_4 (6.0mM)

Malachite Green is a highly potent dye and stains everything it touches. It is imperative that gloves are worn during the preparation of this reagent and that all skin is covered.

2.11.3 – Molybdate Solution

Molybdate solution was prepared consisting of 50.0mM ammonium molybdate dissolved in 3.4M H_2SO_4.

2.11.4 – Measuring Phosphate Liberation

A 24 well plate was prepared with 2 wells containing phosphate liberation buffer alone; one well containing cells at $1x10^6$cells/ml in phosphate liberation buffer and one well containing $2x10^6$cells/ml in phosphate liberation buffer. The plate was incubated at $23^{O}C$ for one and a half hours to allow the cells to adhere to the wells and to allow the cells to condition the buffer with ATP. The plate was then placed on a shaker at 45RPM.

20µl samples were taken from each of the four wells to count as time zero. One of the wells containing only buffer had its sample used as a blank to calibrate the spectrometer. The other was used as the experimental control well to measure phosphate liberation in the absence of cells. 0.5mM ATP dissolved in the phosphate liberation buffer was added to the three experimental wells and samples were taken every three minutes. After a sample was taken it was placed on ice for 15 minutes to limit spontaneous breakdown of ATP.

After the incubation, the samples were removed from the ice and 2µl of Molybdate Solution were added. They were then incubated at room temperature for 10 minutes. Following this 3.71µl of MLG reagent was added and they were incubated at room temperature for another 15 minutes. Finally 20µl of the resulting solution was added to a cuvette and diluted with 480µl of distilled water. This was given a final incubation of five minutes. The absorbance of light at a wavelength 600nm was measured using the spectrometer. It was important that the timings were followed exactly with each sample, as absorbance was observed to increase the longer the samples were incubated, regardless of phosphate concentration.

2.12 – Data Analysis

In science one cannot do an experiment only once and draw conclusions as one cannot tell whether the achieved result arose due to chance. Each of the experiments observing a phenotype, carried out in

this project was carried out a minimum of three times in order to ascertain that the same result occurred each time, often many times more. The exception to this rule was the experiment involving the heat-inactivation of apyrase, which was limited due to the high rate of failure in preparing the protein for treatment.

Some of the results of the experiments are more quantitative. Instead of observing describable differences in the cells the results are numeric, for example: the number of aggregates produced from the same volume of cells under different conditions. For these experiments the results have been given in the form of a graph of an appropriate type for the data. Unless otherwise stated, the graph will typically display the mean data for each treatment, with error bars showing the standard error of the mean of the data for that treatment. The standard error of the mean is calculated as the standard deviation of the data for that treatment divided by the square root of the number of data points generated for that treatment. The number of times the experiment was repeated to generate the data is recorded in the form "n=the number of repeats" in the caption for each graph.

When numbers have been generated by the experiments instead of descriptive data, statistical analysis can be carried out to determine how likely it is that differences in the means of the data sets is due to an actual scientific difference as opposed to random chance. Unless otherwise stated, the statistical analysis on the data sets in the graphs is a Student's T-Test, paired or unpaired depending on whether the data was generated in paired sets. Statistical significance between data points is marked on the graphs by an asterisk (*). One asterisk represents a P value (the likelihood of the difference in means being a result of chance) of less than 0.05. Two asterisks represents a P value of less than 0.01, and three represent a P value of less than 0.001.

Chapter 3 – Investigating The Presence Of Extracellular ATP And The Effect Of Its Removal Upon Growth And Development In *Dictyostelium discoideum*

3.1 - Aim

Initially the aim of the experiments described within this chapter was to determine whether, and under what conditions, *Dictyostelium discoideum* cells secrete ATP. Following the discovery of this, the impact of the removal of this ATP was then investigated.

3.2 – Introduction

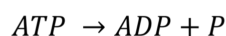
The purpose of the initial experiments in this chapter was to ascertain whether extracellular ATP had a role anywhere in the *Dictyostelium discoideum* life cycle. There is already evidence to suggest that extracellular ATP is released during axenic growth (Parish and Weibel, 1980) and the first experiment carried out was to confirm that this is the case. Experiments were also designed to ascertain whether extracellular ATP was released during the developmental phase of the life cycle.

The presence of extracellular ATP in both instances was determined via the luciferase assay. Luciferase is an enzyme found in fireflies, which reacts with ATP to produce bioluminescence. The reaction is as follows:

$$ATP + luciferase \rightarrow PPi + Luciferase - AMP + O2 \rightarrow AMP + CO2 + Oxyluciferin + Light$$

One of the more useful attributes of this reaction is that the amount of light produced is directly proportional to the amount of ATP present. Unknown concentrations of ATP can be added to solutions and the intensity of the light measured with a photometer (Turman and Mathews, 1996). By comparing this to a standard curve of the light produced by known concentrations of ATP in the same buffer one can deduce the concentration of ATP in said solution (Nichols et al., 1981).

Once extracellular ATP was detected in a particular stage of the *Dictyostelium* life cycle, it was removed from the medium using the potato enzyme apyrase, and the cells observed to see if there was any change in their condition. Apyrase is an ATPase, which catalyses the breakdown of ATP into ADP and phosphate and then ADP into AMP and more phosphate (Riewe et al., 2008).



$$ATP \rightarrow ADP + P$$

$$ADP \rightarrow AMP + P$$

The effect of removing extracellular ATP during the axenic growth phase was investigated by measuring the growth rate. Under normal circumstances cells from the same culture suspended in flasks kept in the same conditions will have the same doubling time. If degrading extracellular ATP were to cause any alterations to the cell cycle, then the timing of the cell cycle phases would differ and as a result the doubling time would change. By comparing the growth curves of normal cells and apyrase-treated cells one could ascertain whether the apyrase was having an effect.

In order to investigate the developmental processes of *Dictyostelium* submerged culture was used. Submerged culture is a method where cells are suspended in a "development buffer" and added to wells of a plate. The cells sink to the bottom of the well, adhere to the base and start to form streams and aggregates. The buffer provides a good medium for the propagation of the cAMP signals while

the clear buffer and plate allow for easy observation of stream formation and aggregates. The major downside of the use of submerged culture is that when cells are submerged in liquid, development does not typically occur beyond the aggregate stage (Sternfeld and Bonner, 1977) and therefore different techniques must be used to observe cells in the later stages of development.

Subsequent to this experiment, investigation was made into the effect of apyrase on *Dictyostelium* growth on agar, firstly to see whether extracellular ATP played a different role depending upon whether the cells were submerged and secondly it was important to see whether this ATP played a role in the later stages of the developmental cycle: slug and fruiting body formation.

3.3 –*Dictyostelium discoideum* Undergoing Exponential Growth Causes Luciferase To Produce More Luminescence Than Untreated Buffer

Shaking culture flasks were set up containing 3ml FM minimal media and *Dictyostelium discoideum* cells seeded at an initial density of $5x10^5$cells/ml. These flasks were shaken at 150RPM for 17 hours. Samples were then taken from these flasks and from flasks of media that had undergone the same conditions without cells. The luciferase reaction was carried out upon these samples. A significantly higher luminescence from a sample would be indicative of significantly higher ATP concentrations within that flask.

In this laboratory HL5 media was normally used for cell culture, but in these experiments FM minimal media was used instead. This was because HL5 is made from yeast extract which is known to have a high ATP content (Min et al., 2006). Use of HL5 would raise the background luminescence and drown out any contribution to extracellular ATP concentrations made by the *Dictyostelium* cells.

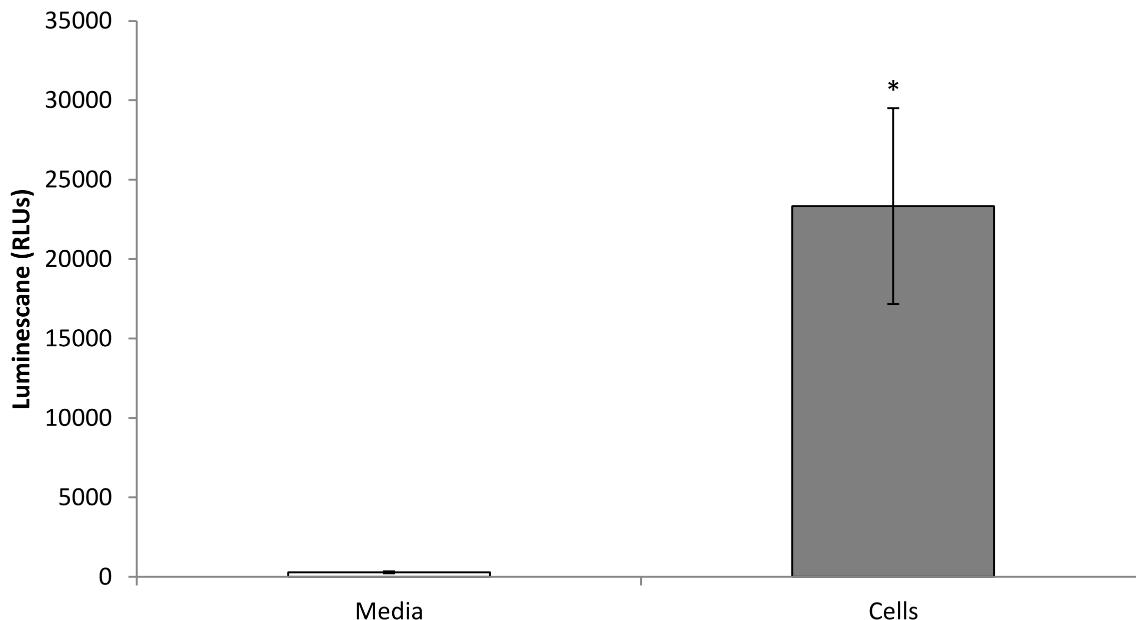


Figure 2 – Increased Luminescence From The Luciferase Reaction In Culture Medium Conditioned by Growing *Dictyostelium*. The luciferase-luciferin reaction results in significantly less luminescence in unconditioned FM minimal growth media than media conditioned with growing *Dictyostelium* ($5x10^5$ cells/ml; 17 hours at 125RPM). The error bars represent standard error of the mean of each data point, N=4, $P<0.05$.

The samples from the flasks that had contained cells produced a significantly higher ($P<0.05$) amount of luminescence than the samples from the flasks without cells (Figure 2). It is important to note that this data does not provide the ATP concentration presents in the media either with or without cells. It merely shows that after 17 hours the buffer containing cells causes luciferase to produce significantly higher luminescence than buffer which does not have cells. This is, however, indicative of there being more ATP present in the cell-conditioned buffer than the un-conditioned buffer.

3.4 –The Addition Of Exogenous Apyrase Has No Effect On Exponential Growth Of *Dictyostelium discoideum*

Having found evidence to suggest that during growth in shaking culture the cells conditioned their media with ATP, the effect of removing this ATP was now investigated. This was done using 4U/ml apyrase.

Cells were suspended in flasks of the FM minimal media at $5x10^5$cells/ml under control conditions or in the presence of 4U/ml Apyrase. The cell density was measured at regular intervals over a period of 54 hours. FM minimal media was again used instead of the HL5 for the same reasons as in the previous experiment.

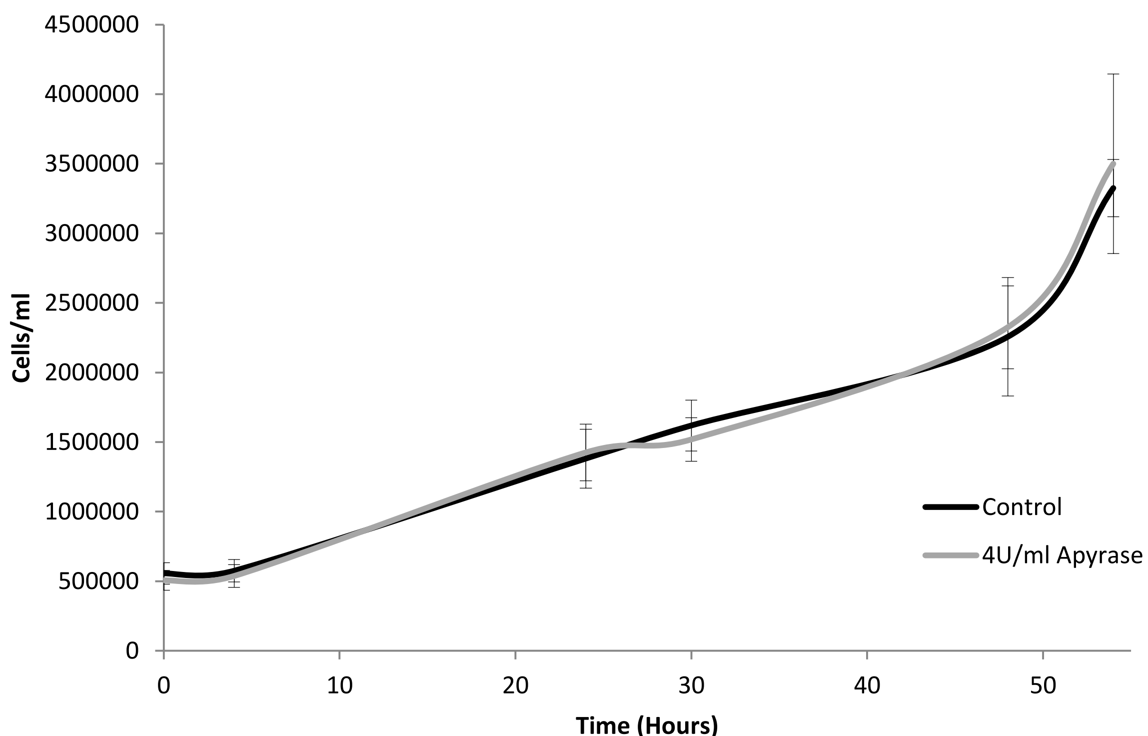


Figure 3 – Growth Curves Of *Dictyostelium.* Growth over a period of 54 hours in FM minimal media under control conditions or in the presence of 4U/ml apyrase. The error bars represent standard error of the mean of each data point, N=4, $P>0.05$.

No significant difference was found in cell density, at any of the time points used, between cells grown under control conditions or with 4U/ml apyrase (Figure 3). Apyrase does not affect the growth rate of *Dictyostelium* within the first 52 hours following seeding of cell cultures.

3.5 - *Dictyostelium discoideum* Secretes ATP During Development

Given the lack of response to removal of ATP during exponential growth the next stage of the *Dictyostelium* life cycle was investigated: development. Again the presence of ATP was investigated using the luciferase assay.

A 24 well plate was set up with wells containing either 1ml of pure development buffer or $5X10^5$ cells and was gently shaken to ensure that any extracellular products of the cells would have an equal concentration throughout the well. Samples were taken of the liquid within these wells every 15 minutes and their luminescence when mixed with luciferase was recorded.

A standard curve of ATP in development buffer was also used to convert the luminometer's readings in RLU into actual ATP concentrations.

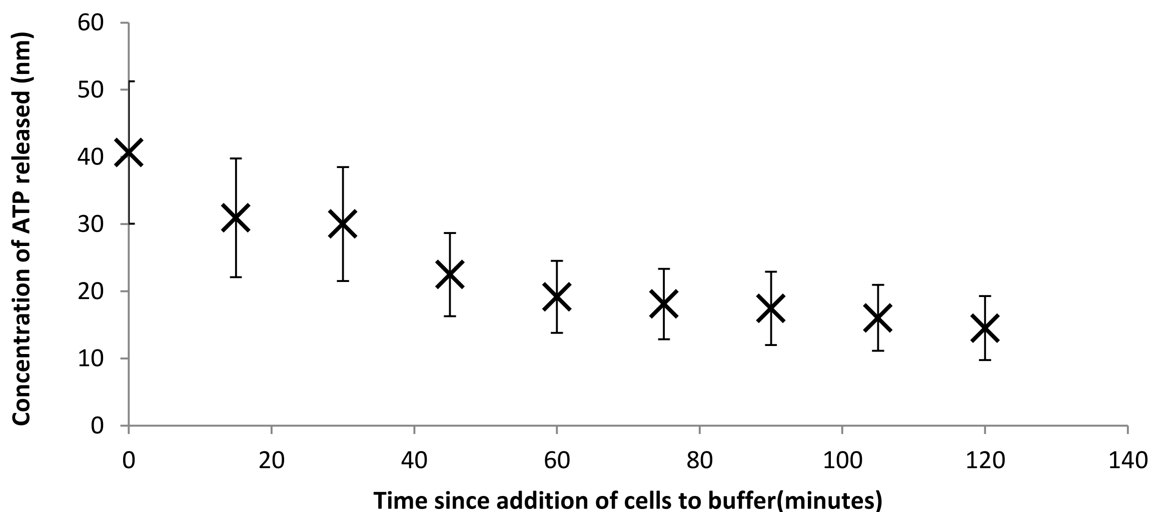


Figure 4 - Detection Of ATP Upon Addition Of *Dictyostelium* To Development Buffer. Mean ATP detected by luciferase-luciferin reaction (luminescence, RLU) over the 120 minutes following seeding of *Dictyostelium* into development buffer at $5x10^5$ cells/ml. Each data point is subtracted from the value obtained at the same time point from buffer that did not contain cells. The error bars represent standard error of the mean of each data point.

Figure 4 demonstrates the change in ATP concentration recorded in the 1ml of development buffer containing $5X10^5$cells compared to wells of pure buffer. Immediately after addition of cells to the buffer, there is a very large change in ATP concentration which implies immediate ATP release. This sudden increase in ATP concentration, though consistently shown in each experiment was highly variable in magnitude. A student's T-Test revealed that over the entire two hour observation period, the ATP concentration was significantly higher than in buffer without cells ($p<0.001$).

Repeated runs of the experiment all showed a high ATP concentration immediately after the cells entered the buffer. The ATP concentration then dropped and levelled off. A two-factor with replication ANOVA test revealed that over the entire two hour period the decrease in ATP concentration was statistically significant ($p=0.01$). On the other hand, if one only takes the data points between 60-120 minutes into account the change over time was insignificant ($p=0.96$), implying that this fall in ATP concentration levelled off at the 60 minute mark.

Extracellular ATP in the presence of *Dictyostelium discoideum* is known to have a short half-life due to the presence of ecto-ATPases (Parish and Weibel, 1980) so the fall in ATP concentration is not unexpected. The fact that the fall stopped, and the ATP concentration levelled off, suggests that the cells were releasing further ATP at a rate proportional to the rate of decay.

3.6 - Submerged Culture

3.6.1 - Submerged Culture - Characterising The Control

Before any effect of the addition of apyrase to *Dictyostelium discoideum* during development could be investigated, the correct experimental procedure as well as the appearance of development under control conditions had to be determined. Submerged culture was prepared in 24 well plates as described in the scientific literature at a density of 1x10^{6}cells/ml. The experiment was set up in the morning and photos were taken over the course of that day and the following morning (Figure 5).

Four hours after addition to the buffer, cells retain their amoeboid shape and show no signs of development	Three hours later there is still no change in cell morphology and no observable signs of development	Development has occured overnight. The cells have formed aggregates

Figure 5 –Development In Submerged Culture. *Dictyostelium* development in submerged culture at cell density of 1x10^{6}cells/ml. Photos taken at 4, 7, and 24 hours after addition to the wells. Images are annotated with descriptions of the developmental process.

It quickly became apparent that, though aggregation occurred over this time period, it was at a rate that was unsuitable for observing the key events. As a result of this it was decided in the second experiment to use a variety of different cell densities as cell density is known to impact the rate of development (Jain et al., 1992).

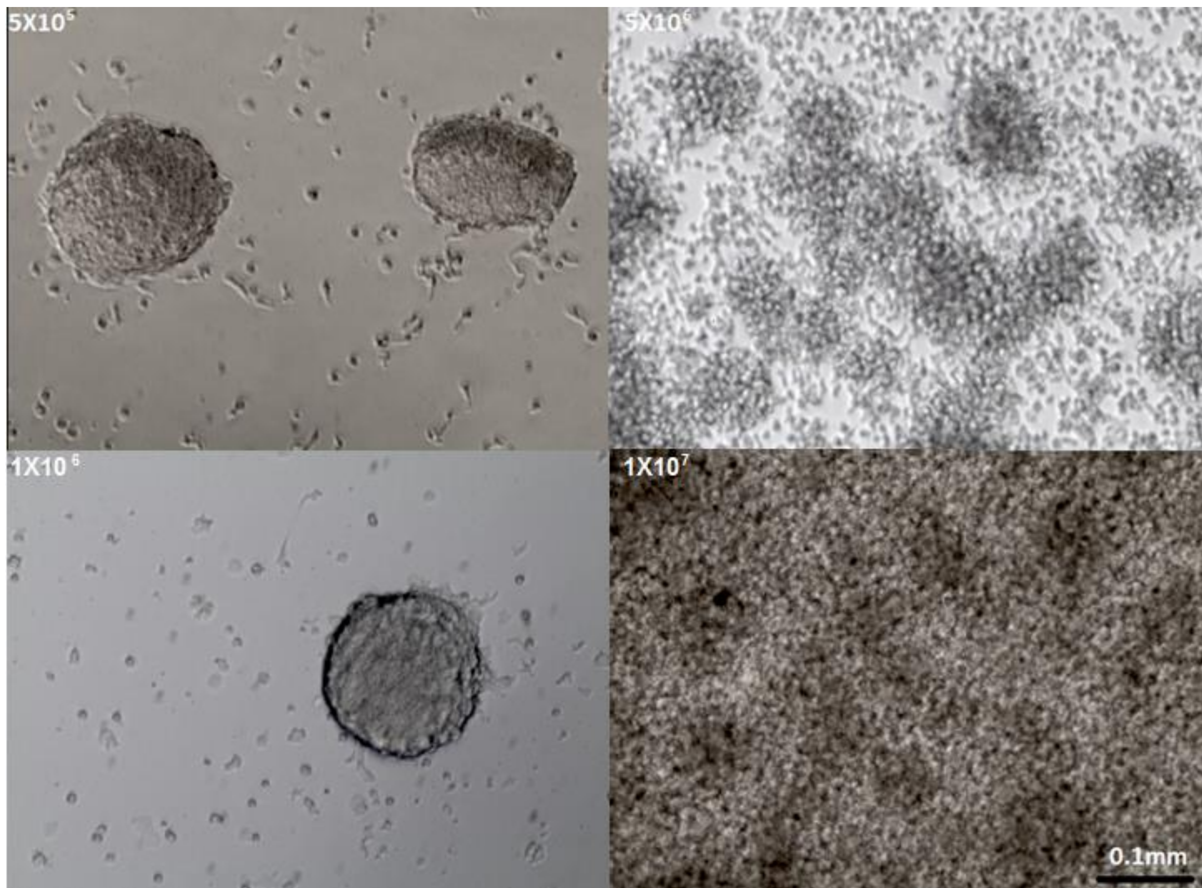


Figure 6 - Developmental Progress Of *Dictyostelium* After 14 Hours At 4 Different Cell Densities (cells/ml). Aggregates of cells are visible at densities of $1x10^6$cells/ml and $5x10^5$cells/ml.

On this occasion the cells developed faster than in the previous experiment. The cells that had been seeded at $1x10^6$cells/ml and $5x10^5$cells/ml had formed aggregates by the time they were observed at 14 hours (Figure 6), whereas the cells at higher densities were already too closely packed together for aggregation to be a meaningful term. Despite this, one could clearly see dark patches where the cells were at a higher density, implying that cells had moved closer together. Carrying out experiments at these higher densities, however, was impractical because these cells did not form noticeable streams.

Attempts were made to perform the experiment at lower cell densities, but below $5x10^5$cells/ml the cells did not develop, even after several days of observation. It is assumed that this was because the cells were too far apart for cAMP waves to propagate.

It was therefore decided to carry out the experiments at densities of both $5x10^5$cells/ml and $1x10^6$cells/ml because, although they had still developed at an impractical rate for observations of all processes to be made, it was hoped that the early stages of aggregation could be visible in $1x10^6$cells/ml and the later ones in the $5x10^5$cells/ml ones. Although the cells at $1x10^6$cells/ml again developed too quickly, this time development of the cells seeded at $5x10^5$cells/ml occurred slowly enough to be observed (Figure 7).

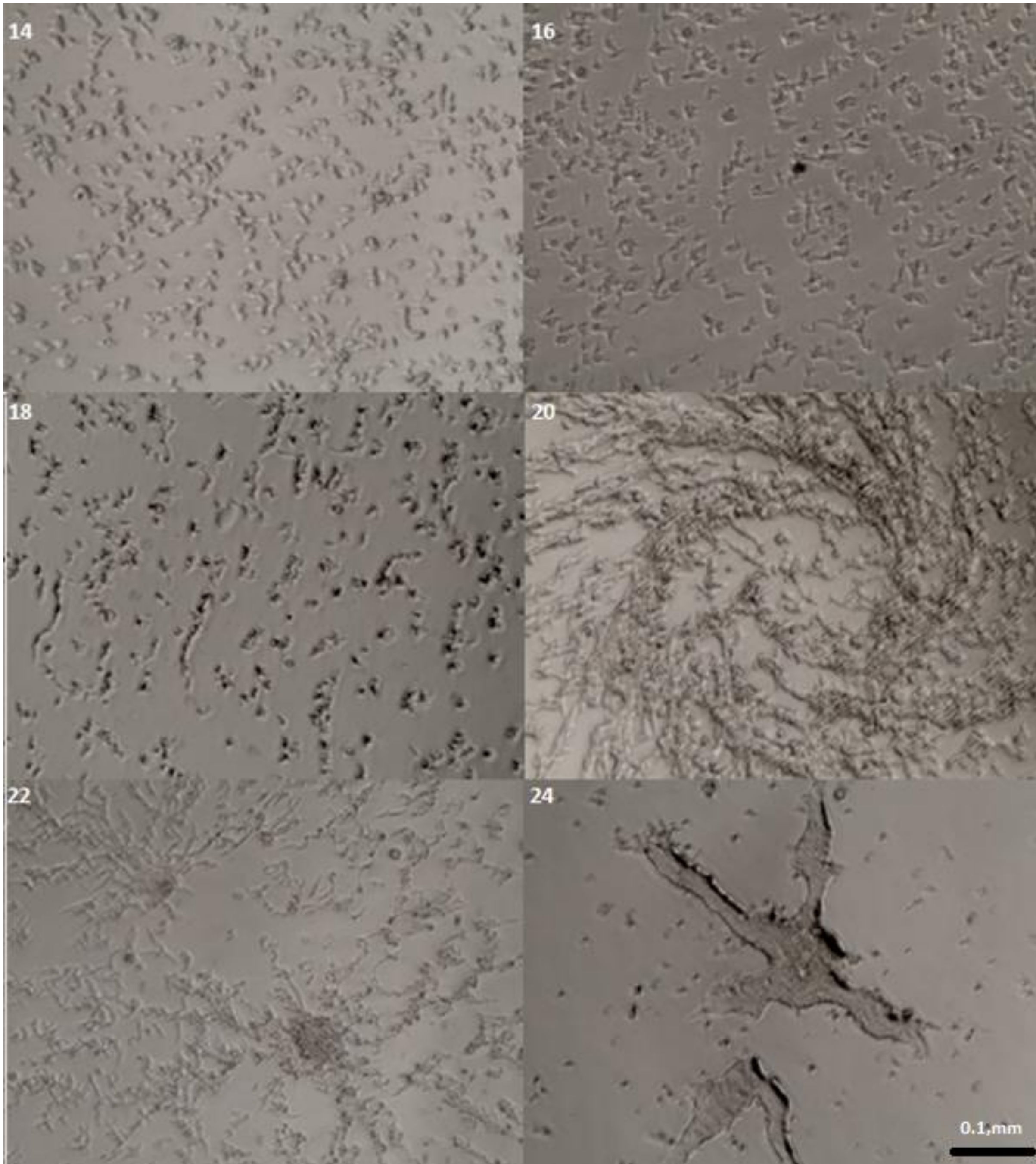


Figure 7 – Development Of *Dictyostelium* In Submerged Culture At Cell Density Of $5x10^5$cells/ml. Between 14-16 hours cells begin to elongate and lose their amoeboid shape. At 18 hours the elongate cells begin to sort themselves into patterns and very early streams, only a couple of cells long, can be seen. By 20 hours the streaming is occurring, the cells are forming a vortex shape as they determine where the eventual aggregation centres will be. These aggregation centres are visible at 22 hours and the cells are moving inwards along the streams towards them. By 24 hours the streams have begun to break up, each segment will form their own separate aggregate.

Dictyostelium is notorious for being highly variable in the timing of developmental events due to minute changes in factors such as laboratory temperature, lighting or cell density of the culture from which they were taken (Jain et al., 1992). Great variation in the timing of development was observed across multiple experiments under what were, theoretically, the same experimental conditions. Investigation showed, however, that the cells in separate wells would start to stream within half an

hour of each other if the cells were taken from the same flask and added to the wells at the same time. Despite the fact that there was great variety in developmental timing between experiments, comparisons could still be made between cells in different wells if they were part of the same experiment.

3.6.2 - Submerged Culture – The Addition Of Exogenous Apyrase To Dictyostelium *Cells Causes A Developmental Delay*

Submerged culture was set up at a density of $5x10^5$cells/ml with two wells containing cells treated with 4U/ml of apyrase dissolved in 20µl of molecular grade water and two wells of control cells containing an equal volume of molecular grade water with no apyrase.

The development of all these cells over a period of fourteen to twenty-four hours was observed. Cells developing under control conditions formed significantly earlier than those treated with apyrase (Figure 8). Even though stream formation did eventually occur, the onset of it was inhibited in some way by the addition of apyrase. It was also observed that, while the cells in the control wells were all at the same stage of development, the apyrase-treated ones were far less uniform with completed aggregates sharing a well with early streams and cells that were still only elongating.

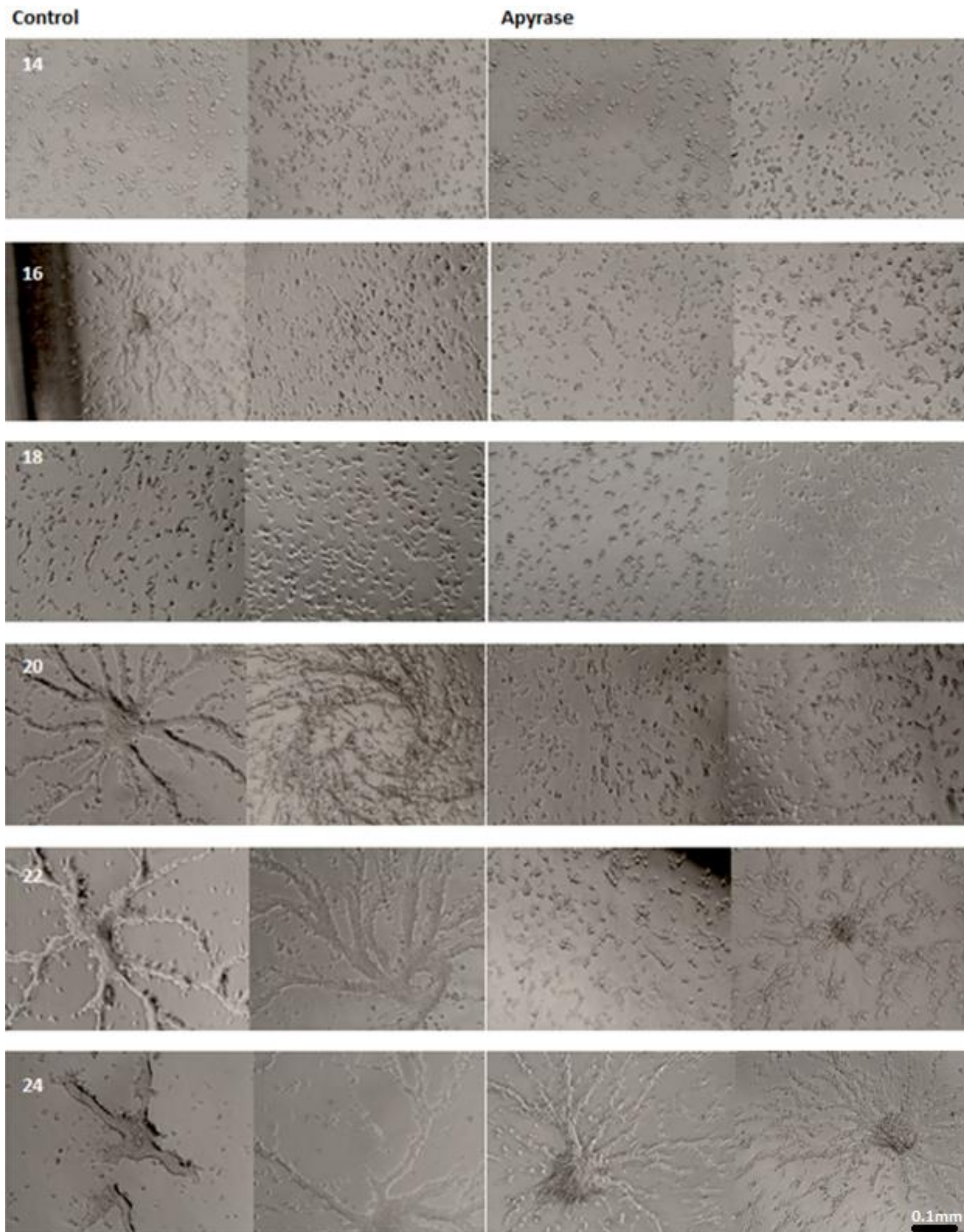


Figure 8 – Apyrase Causes A Delay In The Formation Of Streams. Development of AX4 cells at a density of $5x10^5$cells/ml in cultures treated with 4U/ml of apyrase or an equivalent volume of water over a period of 24 hours. Time since addition to the buffer is given in hours on the left of the image. Photos are of the most developmentally advanced cells found within the well. In control wells streams are first visible at 20 hours and begin to break up by 24 hours. The Apyrase-treated cells, however, take a further two hours before they start to form streams.

It is important to note that if left long enough, the apyrase-treated cells eventually formed aggregates phenotypically similar to the control ones (Figure 9).

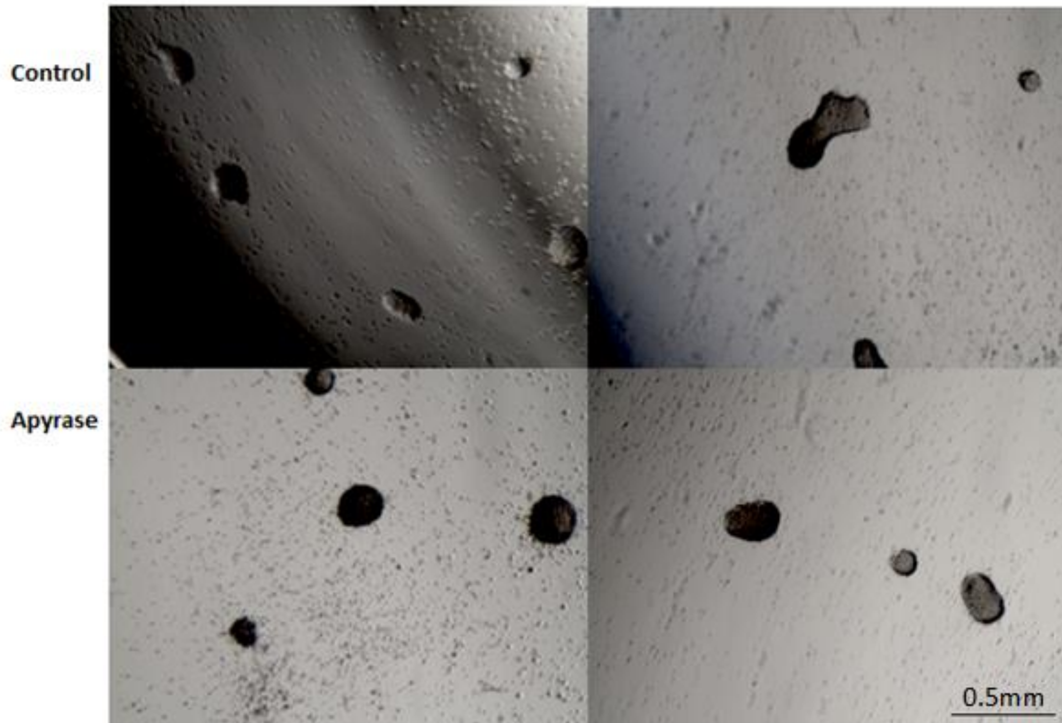


Figure 9 – No Effect Of Apyrase On Complete Aggregate Formation. After 30 hours in development buffer, aggregates of control *Dictyostelium* cells at a density of $5x10^5$cells/ml do not have observable differences to those formed in the presence of 4U/ml apyrase

The most striking effect of adding apyrase to cells was apparent between one to three hours after addition to the buffer. While the control cells did not appear to have changed visibly, the apyrase-treated cells came together in a phenotype that was referred to as "clumping" (Figure 10).

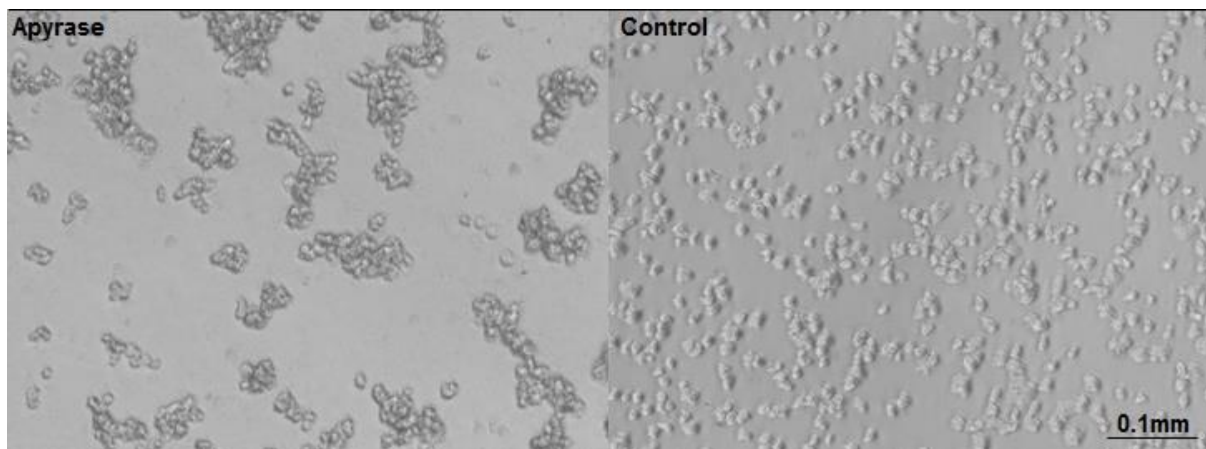


Figure 10 – Apyrase Causes The Formation Of "Clumps". After treatment with 4U/ml apyrase, *Dictyostelium* at a density of $5x10^5$cells/ml form "Clumps." Images taken 1.5 hours after additon of cells to the buffer

Although hard to quantify scientifically, the wells with apyrase added to them appeared to contain a larger number of undeveloped cells following the formation of aggregates (Figure 11).

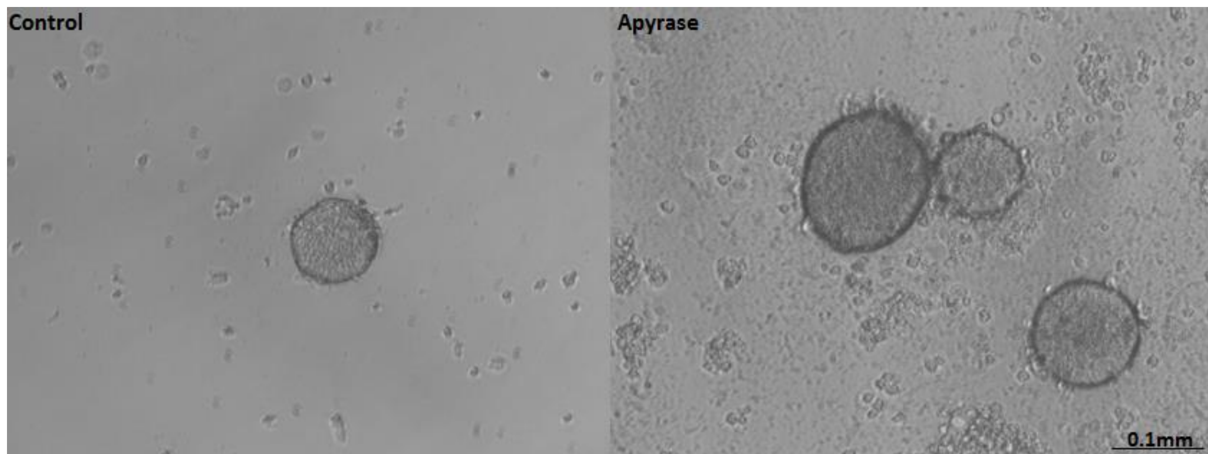


Figure 11 - Evidence For Increased Incidence Of Cells Failing To Commit To Aggregate Formation Upon Treatment With 4U/Ml Apyrase. Cells at $5x10^5$cells/ml. Images taken at 20hours after addition to the buffer.

It was assumed that a larger number of undeveloped cells would lead to the formation of fewer aggregates as this would lead to a functionally lower cell count during development. In order to determine if this was the case the number of aggregates formed in each treatment after 38 hours in development buffer across seven experiments were compared.

There was a large amount of variation in the amount of aggregates formed in each experiment, presumably as a result of the same factors that caused the variation in the timing of stream formation between experiments. In order to be able to combine the data from multiple experiments, each data point was expressed as a percentage of the mean number of aggregates formed in that experiment's control wells. For this data analysis a total of 16 control wells at the $1x10^6$cells/ml density and 17 wells at the $5x10^5$cells/ml density were used. Each was paired with an apyrase well from the same culture of cells and under the exact same experimental conditions (Figure 12).

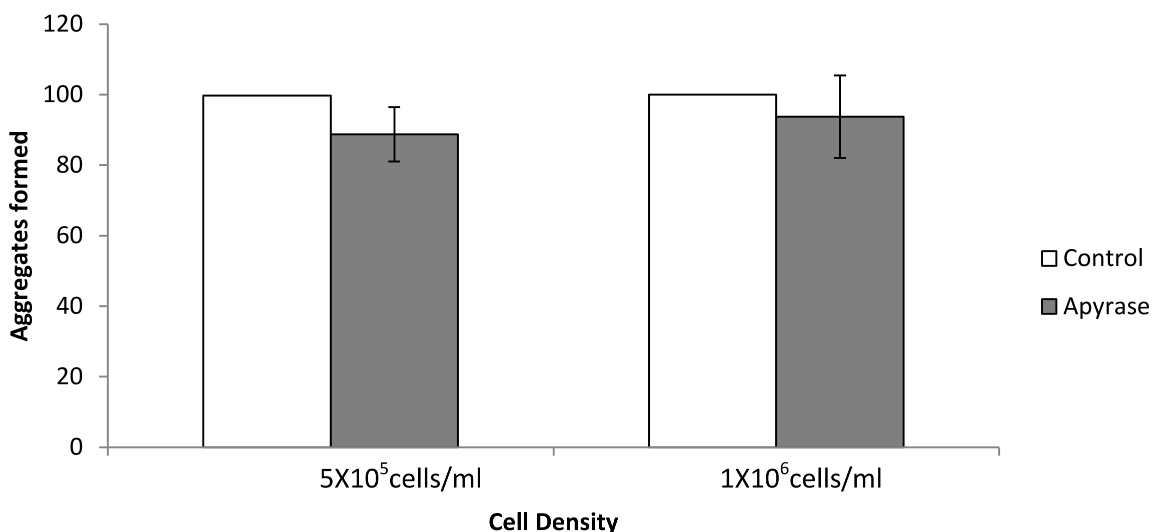
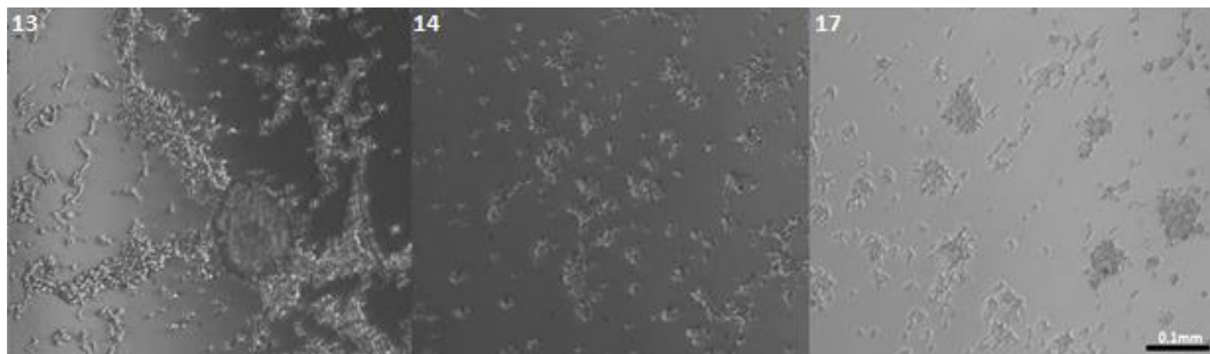


Figure 12 –Apyrase Induced Developmental Delays Caused No Significant Difference In The Eventual Number Of Aggregates Formed. Number of aggregates present in wells containing control and apyrase (4U/ml) treated cells following completion of development at two different densities of *Dictyostelium.* The error bars represent standard error of the mean of each data point, N=7. Each data point is expressed as a percentage of the average amount of aggregates within each pair's control well.

A two-tailed Student's T-Test of this data set confirmed that there was no significant difference between the number of aggregates found in the wells containing control and apyrase-treated cells with P values above 0.05 at both densities (0.61 and 0.34 at $1x10^6$cells/ml $5x10^5$cells/ml respectively).

3.6.3 - Submerged Culture – The Addition Of Exogenous Apyrase Impacts The Development Of Dictyostelium *Cells That Are Already Undergoing Development.*

When 4U/ml apyrase was added to *Dictyostelium discoideum* cells prior to the start of development, they experienced a delay in the formation of streams. This then posed the question whether apyrase would cause the breakup of already formed streams or whether it would only had an effect in the initial signalling phases prior to stream formation. In order to investigate this, 4U/ml of apyrase were added to wells that already contained streams.



Large streams forming throughout the well, before the addition of apyrase | Within an hour the streams have completely broken up | After the break-up of streams, the cells start to form clumps

Figure 13 – Streaming Is Halted By Addition Of Apyrase. Apyrase (4U/ml) was added to *Dictyostelium* cells at a density of $5x10^5$cell/ml half an hour after the starting of stream formation. Time in hours since addition of cells to the buffer is recorded in the top left corner of each image.

The steams very quickly broke up and the clumping phenotype was observed shortly afterwards (Figure 13). This indicates that apyrase had its effect on streams regardless of how far development had progressed at the time of addition. It is important to note that given enough time these cells also eventually formed complete aggregates.

3.6.4 - Submerged Culture – Heat Inactivated Apyrase Is Incapable Of Causing The Apyrase Induced Delay

Having confirmed that adding apyrase to the wells caused a delay in the formation of streams of *Dictyostelium discoideum* cells it was now important to determine whether this was due to apyrase's enzymatic effect or whether it was due to the cells' response to a foreign protein.

Three sets of wells were set up containing firstly cells under control conditions, secondly cells with 4U/ml of apyrase and thirdly cells with 4U/ml of apyrase that had been heat shocked in order to denature it.

The cells that had been treated with heat inactivated apyrase developed at the same time as the control cells (Figure 14), whereas the undenatured apyrase-treated ones still suffered a delay.

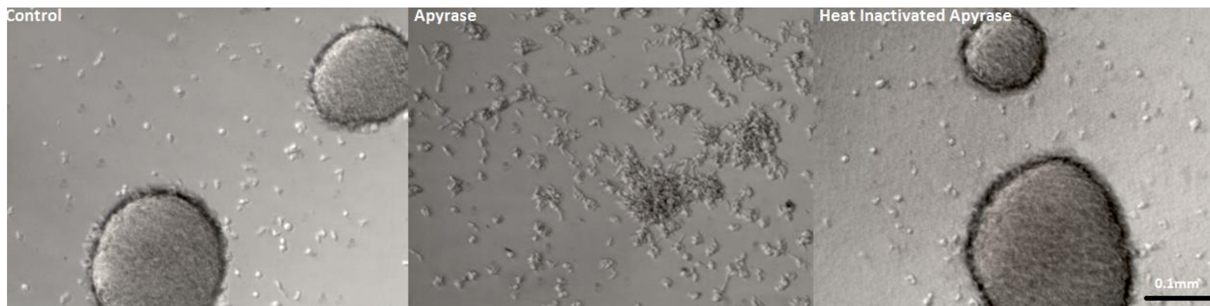


Figure 14 – No Observed Effect Of Heat-Inactivated Apyrase On Development. Cells after 24 hours at $5x10^5$cells/ml under control conditions or with apyrase (4U/ml) that was either enzymatically active or heat inactivated

This experiment proved harder to conduct than expected. When the heat inactivated apyrase returned to room temperature it would coagulate and form a sticky mess, trapping cells and preventing their development. This situation could be slightly improved by lowering the stock concentration of apyrase, but even this was not perfect and many experiments were ruined by this effect. As a result of this, and the relative cost of apyrase, only an n number of two was achievable with non-coagulated, heat treated protein.

3.6.5 - Submerged Culture – The Addition of Extracellular Adenosine Deaminase To Dictyostelium *Does Not Counteract the Apyrase Induced Delay In Stream Formation.*

Apyrase breaks down ATP and ADP into AMP (Fenckova et al., 2011). Though apyrase was shown to cause a delay in stream formation, the question that remained was whether this was truly an effect of removing these nucleotides from the medium or whether it was a result of an increased build-up of the products of ATP hydrolysis. There is no known AMP receptor, but mammalian purinergic systems feature CD73 proteins which convert AMP into adenosine (Colgan et al., 2013). Adenosine is a purinergic signalling molecule with its own receptors (Burnstock and Verkhratsky, 2009) and is known to have developmental effects on *Dictyostelium discoideum* (Jaiswal et al., 2012b). It remained plausible that the apyrase increased AMP production, which in turn led to increased adenosine production, triggering adenosine receptors, which were then responsible for the delay.

Whether increased adenosine production from increased AMP production was responsible for the phenotypes seen was tested by means of adding adenosine deaminase, which breaks down adenosine into inosine (Saboury et al., 2002), to the cells. Four types of well were set up containing cells under control conditions, cells with 4U/ml apyrase, cells with 2U/ml adenosine deaminase or cells with both 4U/ml apyrase and 2U/ml adenosine deaminase. The wells containing only adenosine deaminase were an important control to use. Otherwise, it could be suggested that any phenotype observed in the wells treated with both proteins was a result of extra inosine produced by adenosine deaminase, as inosine is known to affect *Dictyostelium,* though not during development (Parish, 1977).

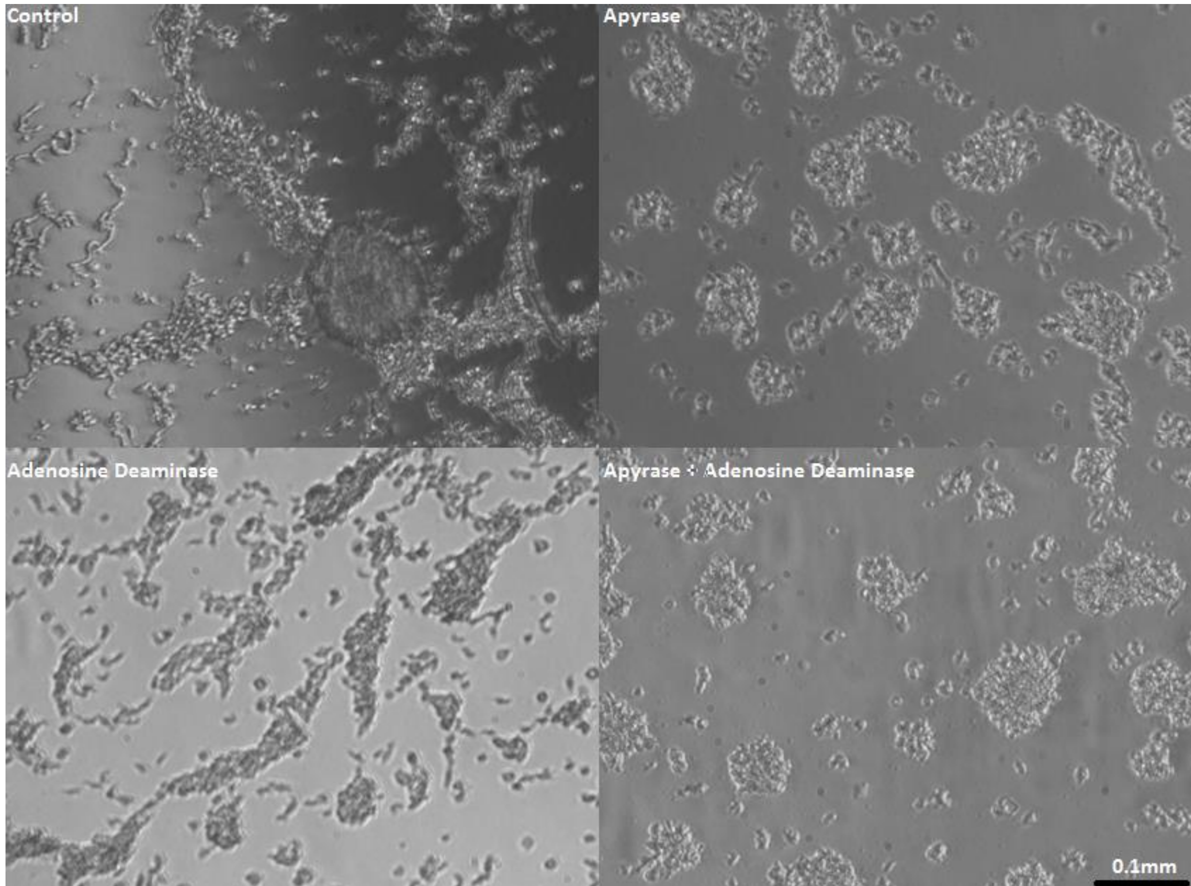


Figure 15 – Adenosine Deaminase Has No Effect On The Timing Of *Dictyostelium* Stream Formation. Photo taken 13 hours after addition to development buffer. In the wells that do not contain apyrase the cells are forming streams. In the wells that do contain apyrase there are no streams present but cells are forming clumps. Cells at a density of $5x10^5$ cells/ml under control conditions or with apyrase (4U/ml) and with or without adenosine deaminase (2U/ml)..

While the control cells and those treated with adenosine deaminase alone began streaming at the same time, all the apyrase-treated cells suffered a delay (Figure 15). This implies that break down of the adenosine building up in the wells of apyrase-treated cells had no impact on the timing of stream formation. Clump formation was also clearly visible in the wells containing cells treated with both proteins.

When aggregation was complete the number of aggregates present in the wells was counted. Curiously, although neither apyrase nor adenosine deaminase on their own caused a change in the number of eventual aggregates formed, when both proteins were added to the cells a significant ($p<0.01$) drop in the number of aggregates was observed (Figure 16).

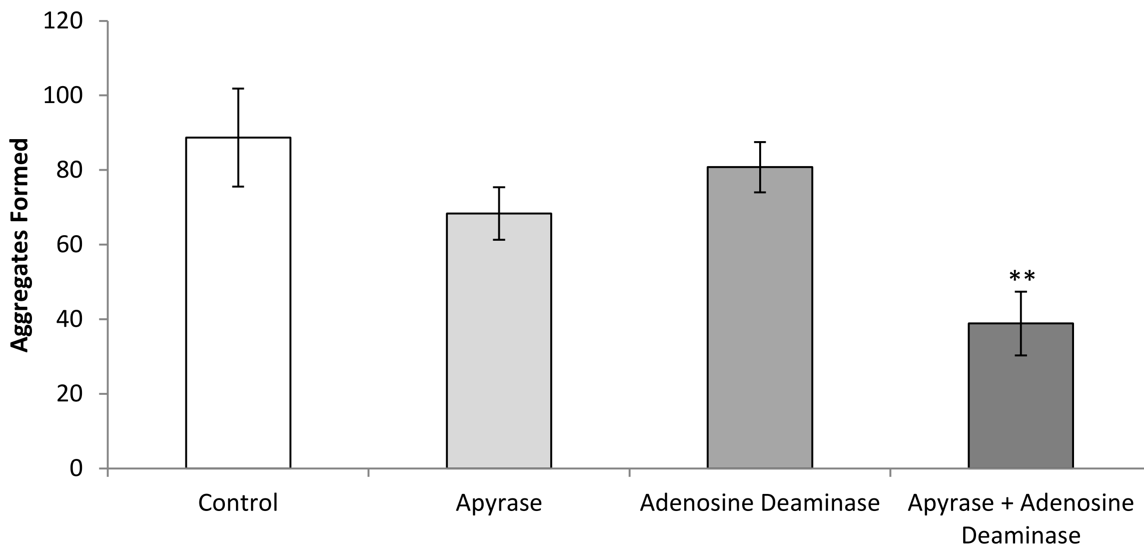


Figure 16 – Additive Effect Of Apyrase And Adenosine Deaminase On Number Of Aggregates Formed During Development. Number of aggregates present in wells seeded with $5x10^5$cells/ml *Dictyostelium* under control conditions or treated with apyrase (4U/ml), adenosine deaminase (2U/ml) or both proteins after a period of 42 hours. The error bars represent standard error of the mean of each data point, N=3 with two wells in each experiment.

The decrease in the number of aggregates produced by the cells that were treated with both proteins was not due to more cells making up each individual aggregate, indeed by eye the aggregates appeared to be a similar size to control aggregates, but was instead a result of the vast number of cells that had not taken part in aggregation. Whether this was due to a failure to express developmental pathways or due to cell death was unclear.

3.6.6 – Submerged Culture – The Effect Of Exogenous Apyrase On Dictyostelium *Development Is Not Limited To The AX4 Strain*

So far these experiments had been carried out purely using the AX4 strain of *Dictyostelium discoideum.* There is, however, evidence that the AX2 strain releases higher concentrations of extracellular ATP during axenic growth than the AX3 strain (Parish and Weibel, 1980) and to have a significantly smaller calcium response to internal ATP than the AX4 strain (Sriskanthadevan et al., 2011). It was, therefore, wondered whether the apyrase effect was also found within AX2, or whether it was unique to AX4. The initial submerged culture development with apyrase was carried out again using AX2 cells (Figure 17).

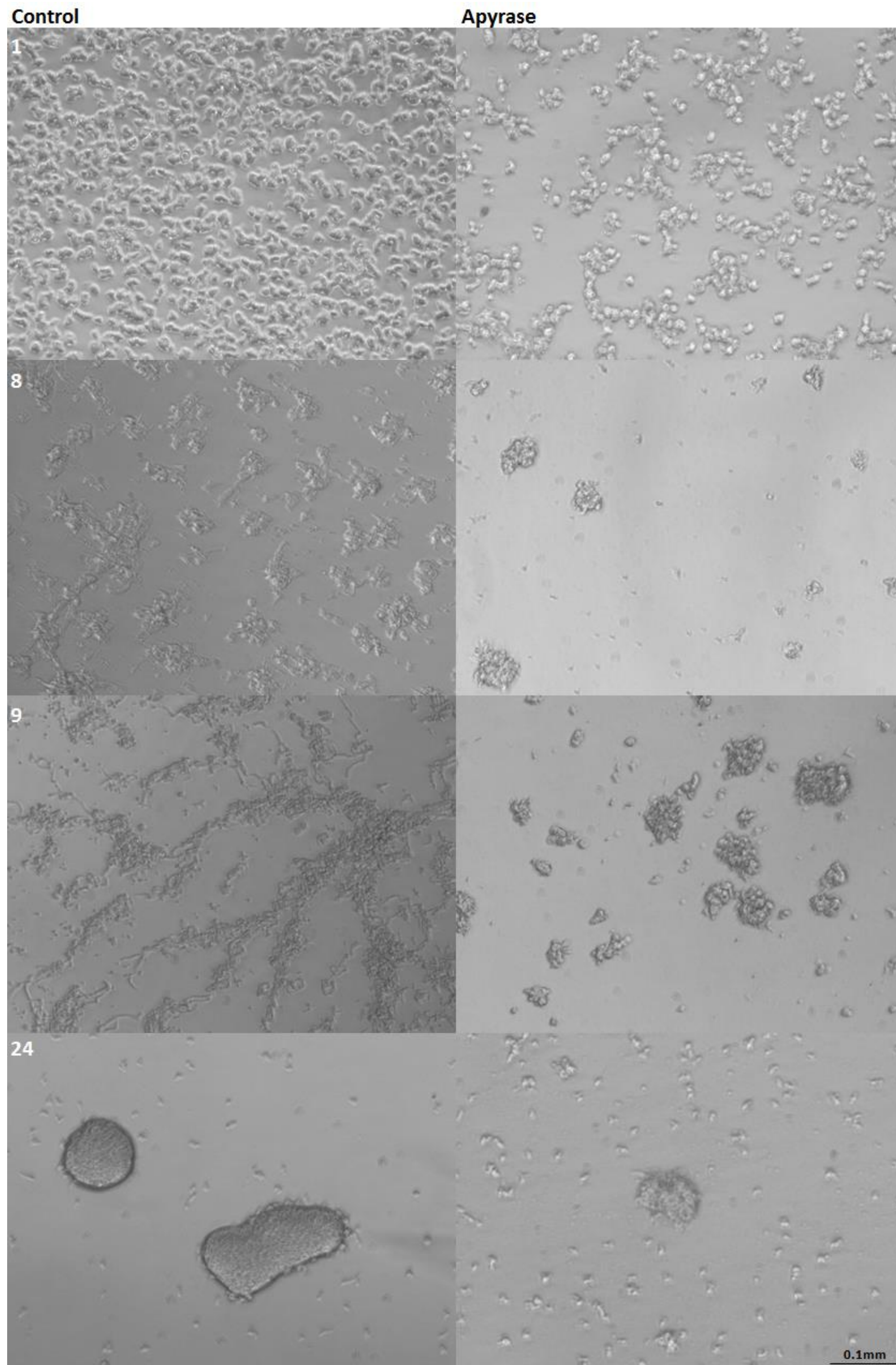


Figure 17 - Apyrase Causes A Delay In The Initiation Of Streaming Of AX2 Cells. Development of AX2 cells seeded at a density of $5x10^5$cells/ml in cultures treated with 4U/ml of apyrase or an equivalent volume of water over a period of 24 hours. Time since seeding is given in hours on the left of the image. Photos are of the most developmentally advanced cells found within the well.

Treatment with 4U/ml of apyrase had the same effect on both strains: the formation of clumps, followed by a delay in the initiation of streaming and the eventual formation of aggregates. It did appear, by eye, that the AX2 cells were forming larger clumps than the AX4 cells, but this is subjective. Furthermore, the large amount of variability in clump size between AX4 experiments makes it hard to determine whether this was an actual phenotypic difference between the two strains.

3.7.0 – Cell Adhesion

Cell-cell adhesion is an important element of *Dictyostelium* development. Cells start to adhere to each other when they first touch within the chemotactic streams and this helps to keep them travelling inwards towards the aggregation centre as well as to form fruiting bodies from the resultant mounds (Noratel et al., 2012).

One suggested explanation for the clumping phenotype seen in cells treated with apyrase was that premature expression of the cell-cell cohesion genes was causing them to stick together during their random movement through the well prior to development. In order to test this an experiment was set up to measure the cohesiveness of cells when they collide during non-developmental conditions (Yang et al., 1997). It was hoped this would provide a method of quantifying the clumping effect and one could then use this to compare the degree of clumping between the AX2 and AX4 cell lines.

3.7.1 Cell Adhesion – Characterising The Control

Initially Eppendorf tubes were prepared containing $5x10^5$ cells in 1ml of buffer to mimic the conditions found within the submerged culture experiments. It was found, however, that at this volume when the cells were inverted there were parts of the side of the tube that were never in contact with the liquid. The clear sides of the tube in these section became cloudy as the experiment continued and it was found that cells were entering these sections and not being resubmerged in the buffer. It was, therefore, decided to increase the volume to 1.5ml in order to ensure that every part of the tube's sides would be submerged with each full rotation. The number of cells and amounts of reagents were therefore scaled up to match this change in volume.

As explained in section 2.10.1 - Aggregation Quotient, for these experiments the data for this section were measured as an Aggregation Quotient. This was a ratio of the number of aggregates seen within a sample of the Eppendorf's volume compared to the combined number of aggregates and lone cells seen. An aggregation quotient of 1 would mean that only aggregates were observed, while a value of 0 would mean that only lone cells were observed.

Initally the experiment was carried out with the cells rotated at 10RPM, but no noticeable change in aggregation quotient occurred under these conditions. Yang et al. used 180RPM for their experiments, but here 46RPM was used. This speed was chosen as it was found to be the highest at which the tubes could be rotated before the centripetal forces pushed the contents of the tubes constantly to the ends and gave a centrifuge-like effect as opposed to a mixing one.

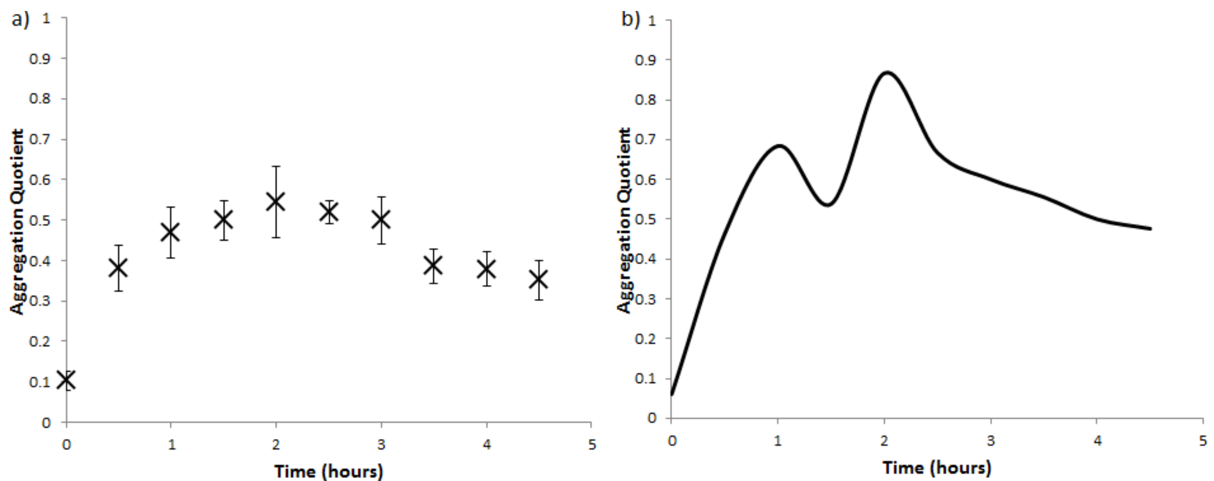


Figure 18 – Aggregation Quotient Changes Over Time Under Control Conditions. *Dictyostelium* cells at a density of 5×10^5cells/ml adhere to each other when mixed in the buffer through chance collisions and the expression of adhesion molecules a) Mean Aggregation Quotient over a period of 4.5 hours. The error bars represent standard error of the mean of each data point, N=8.
b) Representative figure of the change of aggregation quotient in one tube over a period of 4.5 hours

Figure 18 shows a sharp increase in aggregation quotient immediately after the cells are mixed in the buffer. This normally peaked between 1.5 to 2.5 hours and dropped slightly for another hour before levelling off. Experiments where the tubes were left on the rotor for over 24 hours before being monitored again suggested that the aggregation quotient remained at this level. It is important to note that this adhesion in the buffer is independent of cAMP waves as the constant mixing prevents the necessary cAMP gradient from forming.

3.7.2 - Cell Adhesion –Addition Of Exogenous Apyrase To Dictyostelium *Does Not Alter the Aggregation Quotient*

Cell adhesion was measured over a period of 4 hours using cells under the control conditions described above paired with cells in the presence of 4U/ml apyrase. Each experiment included three tubes of each treatment.

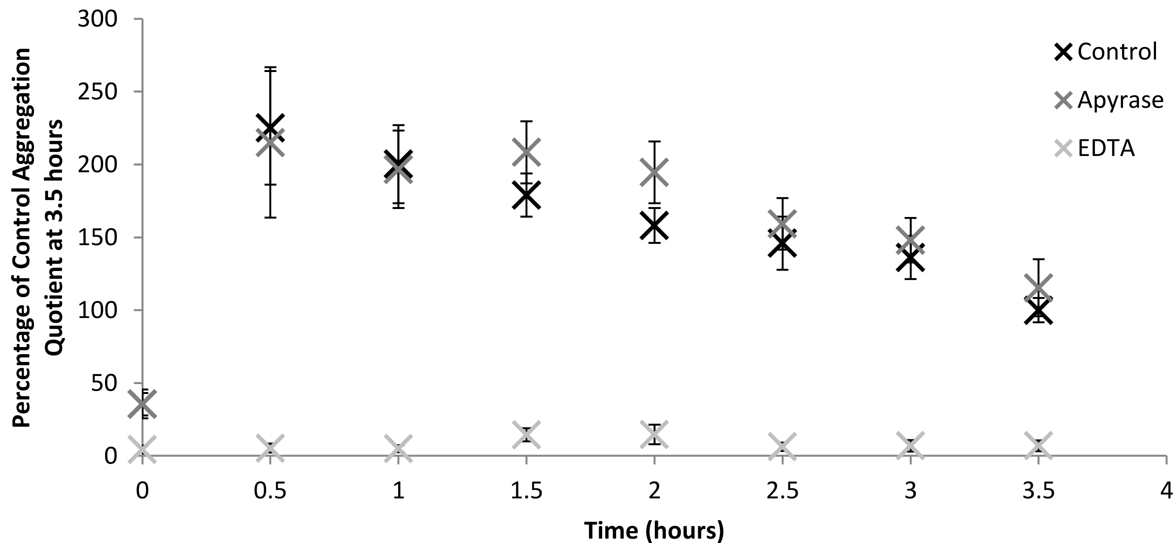


Figure 19 –Addition Of Apyrase Has No Effect Upon The Aggregation Quotient. The change in Aggregation Quotient over time of *Dictyostelium* at a density of $5x10^5$cells/ml is not altered when apyrase (4U/ml) is added. The Aggregation Quotient is, however, altered by addition of EDTA (5mM) which prevents cell-cell adhesion. Control N=15, apyrase N=9, EDTA N=6. The error bars represent standard error of the mean of each data point.

Due to variability in the size and the timing of the peaks in each experiment, each data point was expressed as a percentage of the mean control aggregation quotient at 3.5 hours within the current set of paired experiments. Figure 19 shows that no significant difference was observed between those tubes containing control cells and those containing apyrase-treated cells ($p=0.62$).

In control experiments addition of 5mM EDTA caused a significant reduction in aggregation quotient versus untreated cells ($p<0.001$). EDTA has previously been shown to disrupt cell adhesion in Dictyostelium mediated by the gp24 adhesion molecules which is dependent upon divalent cations (Yang et al., 1997). This acted as a positive control to show that this assay was capable of detecting changes in the adhesiveness of the cells.

With this in mind, it was speculated that cells treated with EDTA and apyrase would still form clumps in submerged culture, which would provide valuable evidence that the clumping effect was not due to the role of EDTA sensitive adhesion molecules. Development was observed in submerged culture at a density of $5x10^5$cells/ml, under either control conditions or with the addition of 5mM EDTA.

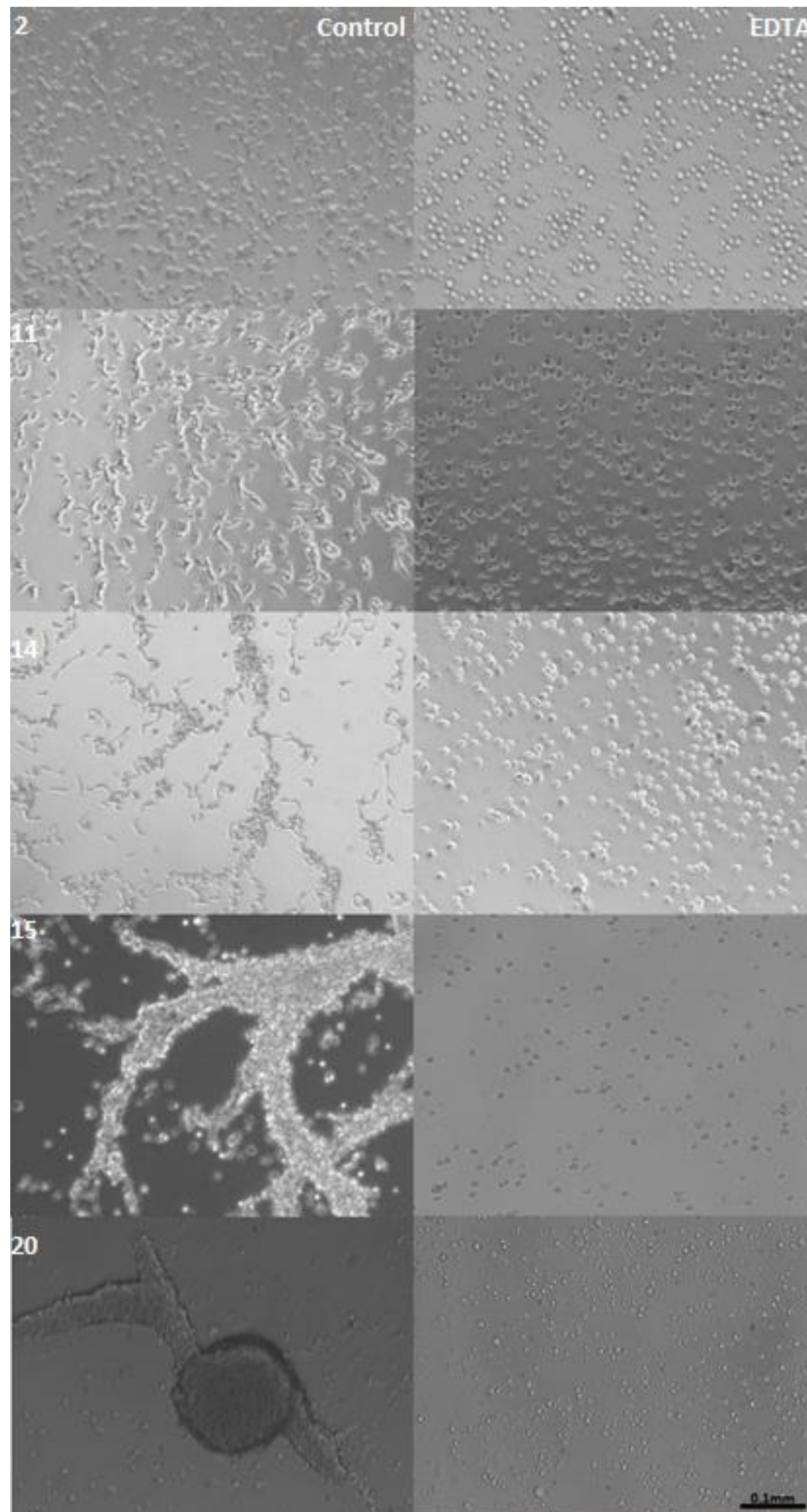


Figure 20 - EDTA Prevents Development. *Dictyostelium* seeded at $5x10^5$ cells in submerged culture over a period of 20 hours either with or without exogenous EDTA (5mM). Control cells start to form streams at 15 hours and by 20 hours are beginning to become complete aggregates. EDTA treated cells fail to show any signs of development.

The cells treated with 5mM EDTA failed to carry out any of the visible stages of the developmental cycle (Figure 20). Cells treated with EDTA did, however, differ from both control cells and cells treated with apyrase in that shortly after addition to the media they appeared to become more rounded

(Figure 21). This occurred even before the control cells had started to elongate and still retained their normal amoeboid shape. This rounded shape was retained throughout the time that control cells developed. It was, therefore, decided that the developmental phenotype of EDTA-treated cells was sufficiently different from control ones that any experiment treating cells with both apyrase and EDTA would give results more characteristic of the EDTA effect than that of apyrase.

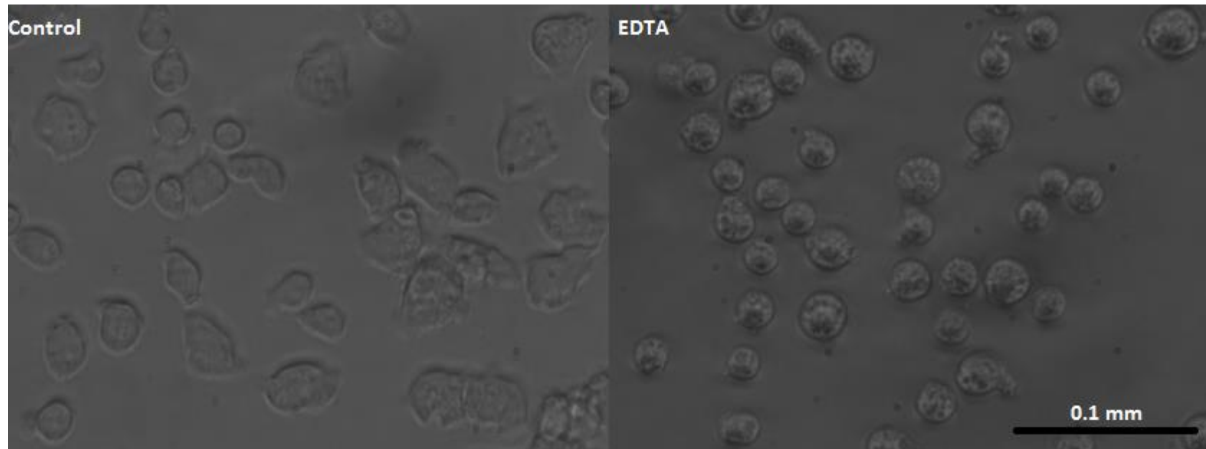


Figure 21 – Rounded Appearance of EDTA Treated Cells. Within 1.5 hours of being added to the submerged culture, *Dictyostelium* seeded at $5x10^5$cells/ml and treated with EDTA (5mM) are noticeably rounder than ones in control conditions

3.8.0 – Development On Agar

Thus far the experiments had looked at the effect of apyrase on *Dictyostelium discoideum* development in submerged culture. This is not, however, the only method of investigating developing cells and in order to ascertain whether apyrase had an effect on the later stages of development the cells had to be observed when developing on agar.

3.8.1 – Development On Agar – The Addition Of Exogenous Apyrase Induces A Developmental Delay In Dictyostelium discoideum *Developing On Agar*

Observing development on agar required larger wells than submerged culture (5cm diameter as opposed to 1cm). Although the cell surface density was kept constant, given that the purpose of this experiment was to develop cells that were not submerged in buffer, they had to be administered to the well at a higher concentration and lower volume (2.5×10^6 cells in 0.2ml of buffer). It was, at this stage, uncertain what volume of apyrase to add to match this alteration in well size. It was decided to initially start with 4U/well and alter it if this proved to not have an effect.

Figure 22 – Apyrase Causes Delayed Development On Solid Agar. Development of AX4 cells seeded at a density of 1×10^4 cells/cm^2 on solid non nutrient agar. The cells treated with 4U/ml water are still at the mound and slug stages of development after 22 hours. At this time the control cells are mostly at the fruiting body stage with some remaining slugs.

This was not necessary, however, as 4U/well was capable of eliciting a delay in development on agar, just as in submerged culture (Figure 22). This effect was most visible after 22 hours, when the control

cells had formed fruiting bodies with a few lingering slugs, while the apyrase-treated ones still mostly consisted of slugs with a few lingering mounds.

Due to the three-dimensional structure of the fruiting bodies it proved difficult to focus on them to take photographs. In one of the control wells a fruiting body happened to form on the side of the well and, although the sideways gravity would affect growth of this fruiting body and it should be discounted as experimental evidence of a delay, a photograph of it is included in the control section of Figure 22 as representative of what was present in the well, but hard to photograph.

3.8.2 - Development On Agar – The Addition of Exogenous Apyrase Decreases the Number of Fruiting Bodies formed by Dictyostelium discoideum

After five days of development, the number of fruiting bodies formed under control conditions and in the presence of apyrase was ascertained. The large size of the wells compared to the field of view under the microscope could render this a subjective measure. To counter this, the fruiting bodies were counted by volunteers who were uncertain which wells contained control cells and which contained cells treated with apyrase. As in previous experiments, due to the large amount of variation between different experiments each data point was expressed as a percentage of the number of fruiting bodies produced in the paired control.

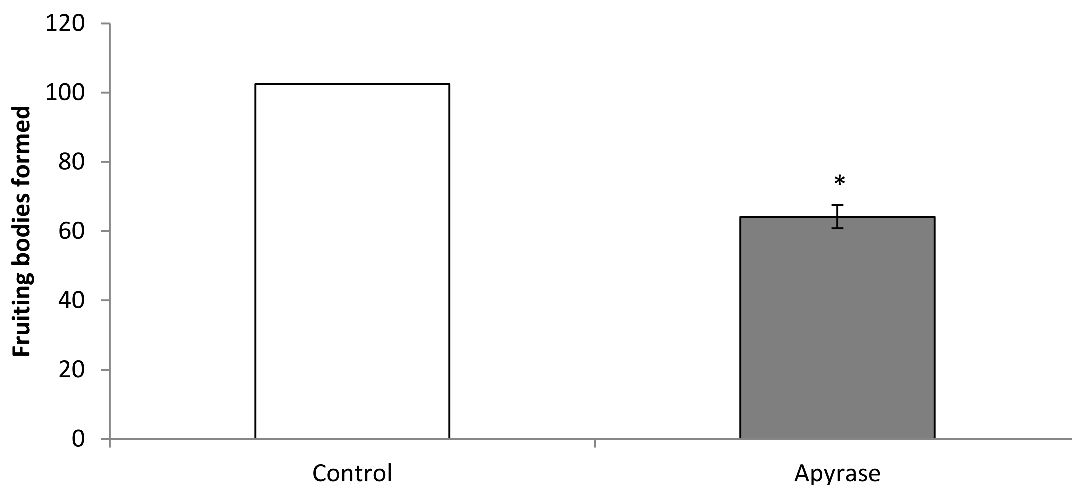


Figure 23 – Apyrase Causes A Decrease In The Number Of Fruiting Bodies Formed. Mean number of fruiting bodies formed by *Dictyostelium* seeded at a density of $1x10^4$ cells/cm^2 on solid non nutrient agar with or without apyrase (4U/well). Each data point is expressed as a percentage of the relevant control. The error bar represent standard error of the mean the data, N=6, two wells/experiment.

The cells treated with apyrase produced significantly fewer fruiting bodies ($p<0.05$) than the cells which developed in control conditions (Figure 23).

3.8.3 - Development On Agar – The Addition Of Exogenous Apyrase Decreases the Number Of Viable Cells Within Fruiting Bodies Produced By Dictyostelium discoideum

The mean size of the fruiting bodies formed by control and apyrase-treated cells was ascertained by taking two fruiting bodies of each treatment and re-suspending them in 4ml of *Klebsiella.* This solution was then spread onto agar plates and the colonies that eventually formed were counted. As in

previous experiments, due to the large amount of variation between different experiments each data point was expressed as a percentage of the number of fruiting bodies produced in the paired control.

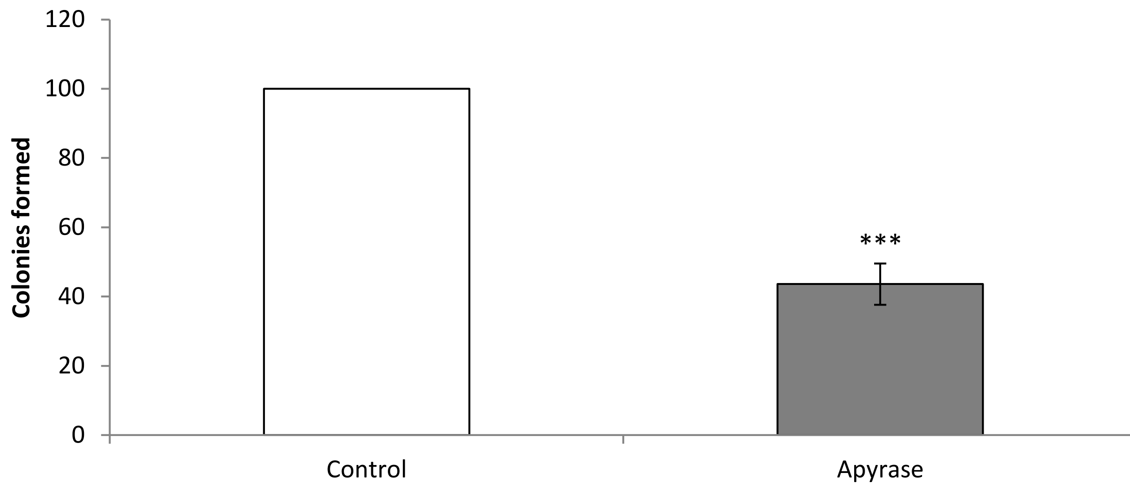


Figure 24 - Evidence A For Smaller Number Of Viable Cells In Fruiting Bodies Developed During Apyrase Treatment. Number of colonies produced by 0.5ml of the mixture of a fruiting body head and 4ml of *Klebsiella*. Spores were produced from by *Dictyostelium* seeded at a density of $1x10^4$ cells/cm^2 on solid non nutrient agar under control conditions or in the presence of 4U/well of apyrase. Each data point is expressed as a percentage of the relevant control. The error bars represent standard error of the mean the data, N=24 plates of control and 28 of apyrase.

The fruiting bodies formed from cells treated with apyrase contained significantly fewer viable cells ($p<0.05$) than those formed under control conditions (Figure 24).

3.8.4 – Development On Agar – Investigating Whether There Is A Link Between Number Of Fruiting Bodies And Number Of cells Within The Fruiting Body Heads

Given that the raw data was easily available from the previous two experiments, it was decided to investigate if there was a link between the number of fruiting bodies produced in a well and the number of colony forming units within each fruiting body head. Given that every well initially contained 2.5×10^6 cells, it seemed possible that the wells with fewer fruiting bodies would have more cells making up each fruiting body.

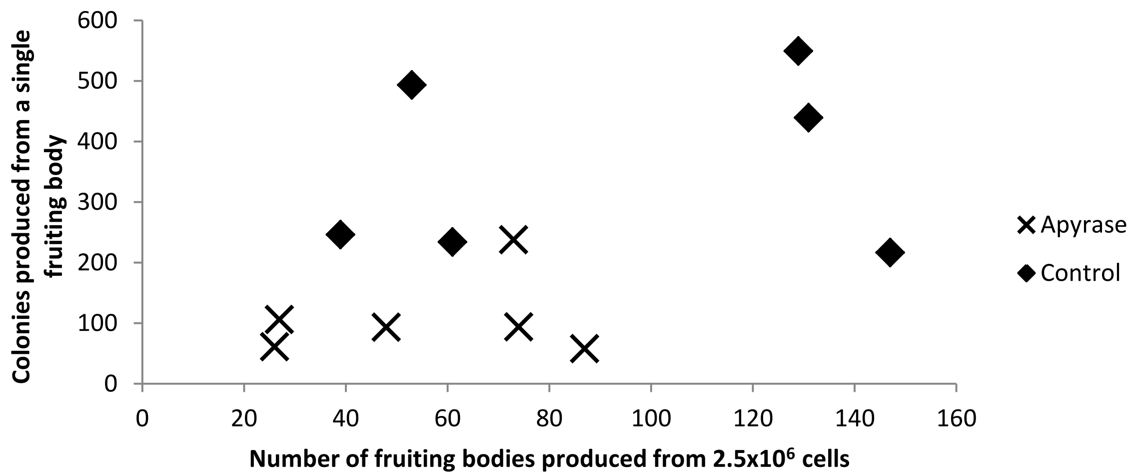


Figure 25 - The Number Of Fruiting Bodies In A Well Containing 2.5×10^6 Cells Plotted Against The Mean Number Of Colonies Produced By Said Fruiting Bodies.

Due to alterations in the set up for the colony counting experiment, only data from the final three of the six agar development experiments were considered. This still provided data on the number of fruiting bodies in 12 wells, as well as the number of colonies produced by four fruiting body heads from each well. The mean number of colonies/fruiting body was plotted against the number of fruiting bodies present in each well (Figure 25).

Considering all twelve data points together there does not appear to be any link between the two sets of data. A Spearman's Correlation coefficient of 0.28 confirms a lack of correlation. When the data is split, to consider data from the control wells and the apyrase wells separately, there is still no link. These correlation coefficients are -0.14 and -0.66 respectively.

3.9 – Conclusions

It is believed that there is a role for extracellular ATP in the regulation of developmental timing in *Dictyostelium discoideum.* The initial evidence for this was in work carried out by Mato and Konijn in 1975 which showed that adding ATP exogenously causes an increase in the number of phosphorylated CAMP receptors. Furthermore, despite the absence of any known extracellular purinergic receptors in this organism, P2X and P2Y-like calcium responses to extracellular ATP were observed by Ludlow et al, in 2008. In this chapter experiments were carried out to investigate the role of extracellular ATP during development.

The results from the luciferase assay suggest that the cells condition their media with ATP while undergoing normal growth. The addition of apyrase to the cells had no effect on the cells' growth

rates. It should, however, be noted that a positive control showing that apyrase was capable of carrying out its enzymatic effect within HL5 media was not carried out. With this in mind it is not possible to conclusively say that breakdown of ATP was occurring under these conditions, one can therefore only definitively state that these results indicate that apyrase has no effect.

If apyrase could indeed be shown to be breaking down ATP under these experimental conditions, with there still being no effect on the growth rate, then one must bear in mind that this experiment would only detect changes in growth rate within the first 52 hours of growth and between the densities of $5x10^5$ and $3.5x10^6$cells/ml. Experiments could not be carried out over longer time periods, which would have allowed the cells to reach higher densities, because the cell cultures became less healthy over time. This was due either to consumption of all the nutrients within the flask of media or to the build-up of bacterial and fungal contaminants. Both of these problems are avoided during normal lab growth by means of regular culturing, but for the purposes of these experiments the cells had to remain within the same flask and volume of media for the duration. This, therefore, provides one possible explanation as to why addition of apyrase caused no observable effect. It might be that the extracellular ATP plays a role later in growth, when the cells reach a higher density. Indeed it is known that at higher densities, the growth rate decreases and the cells begin expressing a different set of genes (Jain et al., 1992). If detection of the high levels of ATP is responsible for this, then no evidence would be detected here when the cells were at lower densities.

It is also important to note that the conditions in which *Dictyostelium* are grown in the laboratory are not the same as the natural conditions in which they evolved. In the wild the environment in which the cells grow is the same as the one in which they eventually develop. The luciferase assay also showed that the cells condition development buffer with ATP and that addition of apyrase at this stage has an effect. It seems plausible that the cells condition their environment with ATP during their growth phase in preparation for the role that it appears to have during development.

These experiments suggest the existence of an ATP signalling system involved in the development of *Dictyostelium discoideum*. Upon entering starvation conditions the cells release, and more importantly maintain the level of, ATP in the medium. Although the ATP concentration measured in these experiments is very small, with a concentration measured in nanomolar, it is known that, in rats at least, prolonged exposure to similarly low levels of ATP will result in the activation of P2X receptors (Roberts et al., 2006). It therefore remains plausible that *Dictyostelium* could detect and respond to the low levels of extracellular ATP that the cells appear to maintain.

The Apyrase used in these experiments breaks down both ATP and ADP at equal rates. These experiments cannot determine whether the effects of apyrase addition are a result of ATP breakdown or ADP breakdown. The luciferase assay suggests the presence of extracellular ATP in development buffer for apyrase to act upon, but extracellular ADP levels were not measured at any point during these experiments. *Dictyostelium* is known to have ecto-ATPase activity but the same effect does not seem to break down ADP (Parish and Weibel, 1980) which would imply the presence of extracellular ADP under control conditions. *Dictyostelium's* calcium response to ATP can be induced equally well by ADP (Ludlow et al., 2009) which implies the two nucleotides activate the same unknown extracellular receptors. Without carrying out experiments in submerged culture using forms of apyrase that are preferential to one nucleotide over the other, one cannot conclusively say whether they both play the same role in these developmental experiments or whether it is removal of one in particular that is responsible for the observed apyrase effects.

Addition of apyrase to submerged culture causes two developmental phenotypes distinct from that of the control: a delay in the formation of streams and the creation of clumps. It remains to be seen whether these two effects of apyrase addition are both a result of the same molecular pathway or instead represent interference with two different ATP/ADP mediated systems. Attempts to heat-inactivate the protein suggest that this makes it incapable of causing the delay in stream formation, further suggesting that these phenotypes are caused by its enzymatic activity rather than the cells responding to the foreign protein. The fact this experiment was only repeated once means that this

result cannot be considered conclusive. Furthermore, the experiments in this chapter suggest that the phenotypes are not caused by the creation of excess adenosine formed from the excess AMP that is the end product of apyrase activity. Removal of adenosine by adenosine deaminase does not restore the control phenotype. One has reason to believe that this adenosine deaminase is indeed enzymatically functional, despite the lack of a positive control, as when it is exogenously added in the presence of apyrase one gets a lower final number of aggregates than otherwise.

There could, however, be products of apyrase other than adenosine which could cause these developmental effects. There are no known AMP receptors (Colgan et al., 2013), but that is not evidence that they do not exist. An as yet undiscovered AMP receptor could be responsible for the apyrase phenotype. The other by-product of apyrase activity is phosphate ions. No reference in the literature could be found to the role of extracellular phosphate ions in *Dictyostelium discoideum* development. Phosphate-containing solutions are included, however, within the development buffer and it seems reasonable to suggest that alterations in the phosphate concentration could be responsible for the developmental delay. The experiment where adenosine deaminase is added as well as apyrase suggests that this explanation is unlikely to be the correct one. One knows that the adenosine deaminase is having an effect due to the alterations in aggregate number. If the apyrase effect was a result of increased phosphate ion generation then one would expect adenosine deaminase, which also produces phosphate as a by-product, to make the apyrase effect more pronounced. No difference in developmental timing between the apyrase-treated cells and the cells treated with both proteins was observed.

Apyrase must be stored at low temperatures and denatures at room temperature (Aldrich, 2009). In these experiments the cells were kept at a constant temperature of $23^{O}C$ within the incubator. It is, therefore, possible that removal of ATP/ADP from the media completely prevents stream formation. Under this hypothesis the eventual stream formation and aggregation found in apyrase-treated cells is not symptomatic of a delay being overcome, but rather the cessation of nucleotide breakdown due to the apyrase becoming denatured. The experiments with heat inactivated apyrase suggest that the enzymatically inactive protein is incapable of delaying stream formation. Evidence to support this hypothesis can also be found in the fact that when apyrase is added to cells that are already streaming, the streaming stops. Alternatively this cessation of streaming in cells which have ATP and ADP suddenly removed may be caused by an abrupt change in gene expression. The hypothesis that the eventual stream formation is due to the apyrase becoming denatured could be tested by taking media samples for the luciferase assay from cells treated with and without apyrase throughout the developmental process. Regardless of whether apyrase becomes denatured during the developmental experiment or not, these results show that the pathway being investigated is important not just for the initiation of streaming, but in maintaining it as well. Shutdown of this pathway causes a rapid halt to streaming.

These experiments suggest that ATP or ADP is needed for aggregation to occur at a normal rate. Furthermore they imply that either ATP/ADP or Adenosine need to be present for healthy aggregates to form. This can be demonstrated by the fact that while apyrase-treated cells had a delay in stream formation and adenosine deaminase treated cells had no observed phenotype, when both proteins were added to the cells there was a large drop in the number of aggregates they were capable of forming. This is curious because a cellular function which requires ATP/ADP or adenosine would be expected to indicate a pathway where ATP is broken down to adenosine. Addition of either purinergic molecule would have the same effect with the only difference being the point in the pathway at which this effect was induced. That the drop in aggregate number is not observed when adenosine is artificially broken down while ATP and ADP are not indicates that this is not the case. This implies the existence of redundant mechanisms to insure that whichever pathway ATP/ADP and adenosine use to regulate aggregate number under control conditions continues to operate in the absence of the detection machinery for either, but not both, of these molecules. This in turn suggests that this pathway is important as *Dictyostelium* has a relatively small genome and there is not much room for superfluous genes.

Further evidence to suggest that this difference in aggregate number is a result of interfering with purinergic signalling can be found in the fact that when cells are exposed to a higher concentration of adenosine than is natural, they form a greater number of aggregates which consist of fewer cells. It is also known that when this effect occurs, there is a greater number of solitary, unaggregated cells (Jaiswal et al., 2012b). It seems likely that this effect and the effect seen in this thesis, when adenosine, ATP and ADP are removed, are related.

Despite the different developmental conditions, apyrase was able to cause a delay in development both in submerged culture and on agar. It is important to note that, while development on submerged culture showed no effects other than the delay, *Dictyostelium* development on agar produced significantly fewer fruiting bodies when treated with apyrase. This could be explained by either a fundamental difference in how development occurs in the two forms of culture or it could indicate that the apyrase-treated cells have difficulties in moving from the aggregate stage of development to the fruiting body stage. Testing this second hypothesis proved difficult as aggregates are not visible during development on agar. The number of mounds formed could not be used as a reliable method of determining aggregate number, as the number of mounds present at a given moment varied considerably over the time period of one experiment.

It is known that there is a certain minimum size a fruiting body must attain, otherwise it collapses (Jang and Gomer, 2008). When the fruiting bodies were spread on bacterial plates, the ones formed by apyrase-treated cells only produced 40% of the number of colonies produced by control ones. This indicates that there are fewer viable spore cells within the apyrase-treated fruiting body heads, though whether this represents fewer cells overall or merely an increase in non-viable cells or stalk cells could not be determined. The fact that there fewer fruiting bodies formed when treated with apyrase and the observation that apyrase seemed to cause fewer cells seemed to join aggregates in submerged culture both provide valuable indirect evidence to suggest that apyrase decreases the number of cells that commit to aggregation.

It was hoped, given that the number of cells present in each well remained consistent, there would be a correlation between the number of fruiting bodies present and the number of cells within them, and that apyrase would impact said correlation. There did not, however, seem to be such a correlation, perhaps implying that in every well there were always cells that did not commit to aggregation. Alternatively this lack of correlation is perhaps just indicative of natural variation in the exact numbers of cells plated into each well.

In these experiments the presence of apyrase also induces the formation of “clumps” of cells. It was initially believed that these clumps were a result of premature expression of cell-cell adhesion molecules and this was measured using an already existing protocol. This protocol did not, however, detect any impact of addition of apyrase. The experiment did, however, seem able to detect changes in cell-cell adhesion as demonstrated by adding EDTA and observing the rapid fall in Aggregation Quotient.

These results, however, do not necessarily disprove the idea that these clumps are caused by cell-cell cohesion, it only proves that clumps did not form under these experimental conditions. Despite the fact that the cells were kept in the same buffer as in the submerged culture experiments, the environment was still very different. In the adhesion experiment the cells were rotated constantly. This prevents development occurring because the mixing ensures an even distribution of cAMP and cAMP-degrading PDE and prevents the formation of a gradient. If the clumps indeed consisted of cells adhering to each other then one could still get the results seen in these experiments. This would mean that Clump formation is dependent upon the expression of the developmental gene pathways which are triggered only once the cAMP waves are detected. It could be definitively confirmed

whether this was the case with an investigation into the mRNA expression of the cell-cell adhesion genes, such as *cadA,* during development in control conditions and with apyrase.

An alternate explanation for the presence of the clumps could be that, rather than being cells sticking together, they are cells that have chemotaxed close to each other, causing a high density of cells in one area. Interestingly this is in some ways similar to the phenotype predicted for situations in which there is an increased amount of secreted cAMP-degrading phosphodiesterase compared to the membrane bound form. In this scenario, the creation of too many cAMP waves cause constructive interference and as a result many competing aggregation centres form (Palsson, 2009). It is not being suggested that the same effect is occurring in these experiments. In the simulations there this caused a in the number of aggregates formed, which was not the case in these experiments, but something similar could be occurring with many cells coming together too quickly and uncoordinatedly and then having to re-sort themselves afterwards before they can form streams.

It is unknown whether the cells treated with apyrase form clumps when they develop on agar, as the individual cells cannot be visualised in these experiments. Perhaps a more powerful microscope could resolve this problem. If indeed it turned out that clumps did not form on agar, even though cells which developed on agar experienced the delay, it might lend credence to the idea that clumps are a separate phenotype from the delay.

Chapter 4 – Investigating Ecto-ATPases And Their Role In Regulating Development In *Dictyostelium discoideum*

4.1 – Aim

The luciferase assay had shown the presence of ATP in the extracellular medium during development and suggested that artificial breakdown of ATP and ADP by apyrase impacted the developmental processes. This implied the existence of a purinergic signalling system involved in the regulation of *Dictyostelium discoideum* development. So far this hypothetical system had only been investigated by observing the phenotypes when ATP/ADP signalling was prevented. In this chapter investigations were begun into ascertaining how this system is regulated under normal conditions.

As of the time of writing there are no known ATP/ADP secretion or detection systems within *Dictyostelium discoideum,* but it is known to have methods of breaking down extracellular ATP to ADP. This is, therefore, the most promising place to start investigation of this hypothetical purinergic signalling system. In this chapter experiments were carried out to measure *Dictyostelium discoideum's* natural capability to degrade ATP and whether developmental phenotypes could be induced by interfering with this.

4.2 - Introduction

There is evidence for the existence of a *Dictyostelium discoideum* cell surface enzyme which normally keeps the extracellular ATP levels in a steady state (Parish and Weibel, 1980). Experiments carried out by Parish and Weibel show that it is sensitive to Suramin, a common ecto-ATPase inhibitor which also has an inhibitory effect on P2 receptors (Chen et al., 1996), and to the local concentration of Mg^{2+} ions. If a way of measuring this protein's activity could be devised, then it would be possible to deduce the concentrations of these factors that would affect the cells in the submerged culture conditions. This would allow more fine-tuned interference with the ATP signalling system.

Initially a modification of a phosphate liberation assay used to detect ecto-ATPases in *Acanthamoeba* (Sissons et al., 2004) was used to determine whether the cells have their own ecto-ATPase activity. Some of the modifications made when the assay was used in other organisms (Feng et al., 2011) were also taken into account during experimental design. This experiment works by measuring the changes in phosphate concentration following the addition of exogenous ATP, using the assumption that the phosphate is produced from ATP breakdown. It is assumed that if a greater rate of Phosphate liberation occurs in the presence of cells than in buffer alone, it is indicative of ecto-ATPases breaking down ATP quicker than the background rate. Despite the fact that Sissons, Feng and Ludlow all take such results as evidence of ATPase activity, this experiment cannot differentiate between ATP breakdown and other sources of phosphate release.

If the assay gave results indicative of the activity of *Dictyostelium discoideum* ecto-ATPases, then the next stage would be to try and identify their inhibitors as a way of characterising them. It was not immediately assumed that common ecto-ATPase inhibitors would be effective, due to the fact that other elements of this hypothetical ATP signalling pathway, such as the P2X-like receptor, have already been shown to not be affected by common inhibitors (Ludlow et al., 2009).

If ecto-ATPases could be shown to be present, it could be inferred that their function is to break down extra-cellular ATP. The rate of this breakdown was earlier implied to be critical for ensuring the

correct timing of developmental events and the makeup of fruiting bodies. This could be tested by treating the cells with inhibitors of the ATPase activity and observing whether this caused an effect on development. As the addition of extra ATPases (apyrase) caused a delay in stream formation, it was anticipated that inhibition of ATPases would cause an effect as well. One could be tempted to assume that ATPase inhibitors would provoke the opposite effect to apyrase, but biological systems are highly complex and calibrated precisely and it was far more likely that any human interference of the ATP signalling system would result in negative effects on the cells.

4.3 – Phosphate Liberation

4.3.1 – Phosphate Liberation – Measuring Change In Absorbance In Control Conditions

In these experiments wells of a 24 well plate were set up containing either $5X10^5$, $1X10^6$ or 0 cells in 1ml of Phosphate Liberation Buffer. These plates were left for 90 minutes to allow cells to adhere to the sides of the wells and condition the media with secreted compounds. Then 0.5mM ATP, dissolved in Phosphate Liberation Buffer, was added and the wells were gently shaken for the next 6 minutes while samples of the medium were taken. Absorbance of light with a wavelength of 600nm through the supernatant of these samples was measured using a spectrometer. A change in the absorbance of light is indicative of a change in phosphate concentration and this is often assumed to be a result of ATPase activity (Sissons et al., 2004).

A calibration curve of phosphate concentration was not drawn for these experiments. This is because, while the results within a single experiment remained with a similar range, there was large variation in the readings gained between different experiments. The most likely explanation for this is that the buffer had to be freshly made up for each experiment. Slight variations in buffer composition, as well as similar slight variations of the concentration of ATP added to this buffer daily, gave very different readings, even though the pattern of data remained the same. This means that the data for these experiments could not presented as a change in phosphate ion concentration, but is instead given as the change in absorbance.

This large variation in the numbers gained between experiments also made initial attempts at interpreting the data difficult. In order to minimise this variation between experiments, each data point is shown as the difference between the absorbance at that specific time and the absorbance of a sample taken from the same well at time zero. As this would render each data point at time zero as having a value of zero with no variation, the data points for this time point have not been shown in the following graphs and only the data obtained after three and six minutes is displayed.

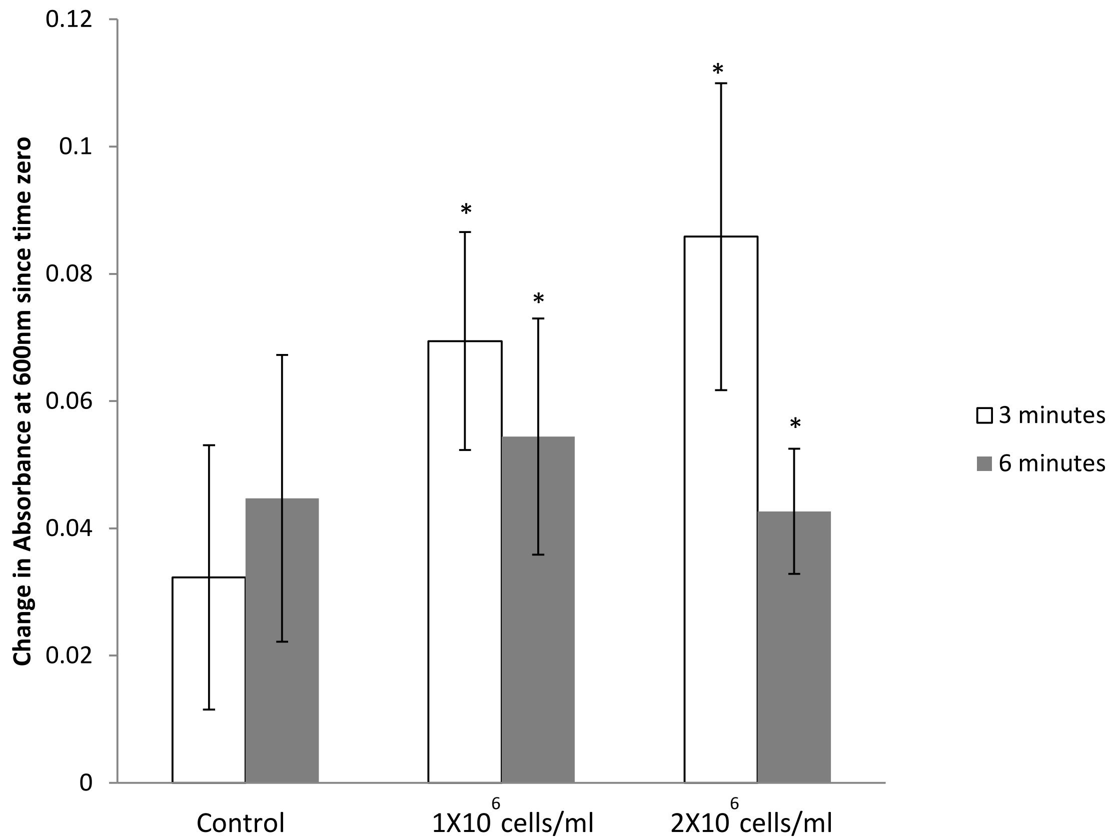


Figure 26 – There Is Significant Change In The Absorption Of light From Samples Taken From Wells Containing Cells After Addition Of Exogenous ATP Between Time Zero And Either Three Minutes Or Six Minutes. Data at three minutes is significantly different in each of the samples from wells containing cells from the control wells at the same time point ($p<0.05$). There is no statistical difference at six minutes between the wells containing cells and the control that do not, but the wells containing cells still have significantly more absorbance ($p<0.05$) than the same well at time zero. Data points taken at 3 and 6 minutes. The error bars represent standard error of the mean of each data point, N=7

A paired T-Test of the values obtained at time zero and three minutes after addition of the 0.5mM ATP (Figure 26) found significant difference ($P<0.01$) in the absorbance of 600nm light in the samples that came from wells containing cells. There was no significant difference found in the readings obtained between these two time points in the control wells. There was also a significant difference ($P<0.05$) in the absorbance of 600nm light in the samples that came from wells containing cells between time zero and six minutes. Again there was no significant difference between readings obtained between these time points in the control wells.

There was also statistical difference in the change in absorbance at three minutes between the wells containing cells and the control ($p<0.05$ in both instances). Interestingly there was no significant difference between the absorbance in wells containing cells and the control at 6 minutes, and indeed by looking at the graph one can see at both cell densities the change in absorbance since time zero has fallen to be at a similar level to the control by this time.

A change in absorbance is indicative of a change in phosphate ion concentration (Feng et al., 2011). The most likely source of phosphate ions in this situation would be from the breakdown of the

exogenous ATP. The significant difference observed at three minutes between the change in absorbance in the wells containing cells and those that do not could therefore be indicative of ATPase activity. This fails to account for the fact that there is no significant difference observed at six minutes.

4.3.2 – Phosphate Liberation – Measuring Change Of Absorbance In The Presence of Suramin

Unfortunately there are no known specific inhibitors which only impact ecto-ATPases (Knowles, 2011). Suramin is known to inhibit both mammalian ecto-ATPases (Chen et al., 1996) and *Acanthamoeba* ecto-ATPases (Sissons et al., 2004), so it seemed a suitable first choice for attempting to ascertain a compound that would inhibit the *Dictyostelium* protein. The previous experiment was repeated, but this time each well also contained 250µM suramin. This concentration of suramin was chosen as it was known to be sufficient to inhibit the ecto-ATPases of *Acanthamoeba*, the most similar organism to *Dictyostelium* upon which this experiment had already been carried out.

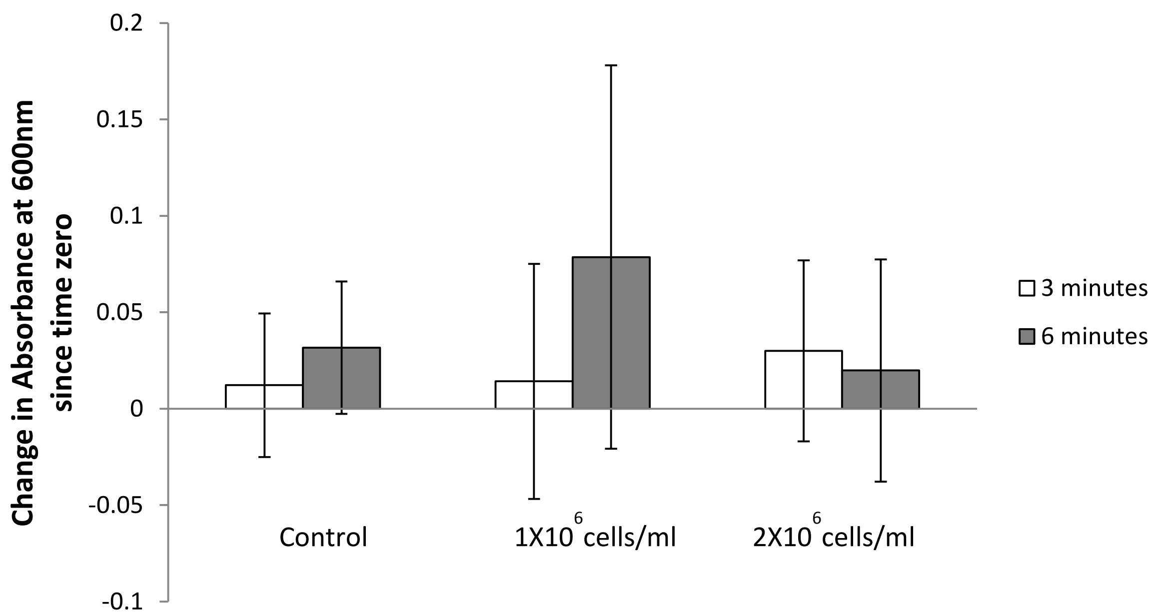


Figure 27 – In The Presence Of Exogenous Suramin, Exogenous ATP Causes No Alteration In Absorbance At 600nm In The Six Minutes After Addition. Addition of ATP (0.5mM) in the presence of suramin (250µM) causes no significant difference in absorbance of light at a wavelength of 600nm. Data points taken at 3 and 6 minutes. The error bars represent standard error of the mean of each data point, N=6

In these experiments there was no significant difference found in the change of absorbance at zero minutes and either three or six minutes after addition of ATP in any of the wells (Figure 27). There was also no significant difference observed in the change in absorbance in samples from wells containing cells and control wells.

It had previously been suggested that, in the absence of suramin, cells cause an increase in phosphate ion concentration three minutes after 0.5mM exogenous ATP is added. This experiment indicates that, whatever was responsible for the significant differences observed in Figure 26 is inhibited by the addition of 250µM suramin.

It is unfortunate that, due to the labour intensive nature of these experiments, it was impossible to carry them out for longer than six minutes in order to ascertain whether one would ever observe a change in absorbance.

4.3.3 – ATPase Activity – Measuring Change Of Absorbance In The Absence Of Mg^{2+} Ions

Many ATPases are dependent on Mg^{2+} ions for their operation and these are present in both the phosphate liberation buffer and the development buffer. As well as testing if inhibition of ecto-ATPases by removal of suramin elucidated the data it was also tested whether removing Mg^{2+} ions from the buffer would have similar results. This time the 2mM MgCl was left out of the buffer preparation. It was hoped that the Mg^{2+} ions would not prove necessary for the MLG-phosphate reaction and this did appear to be the case.

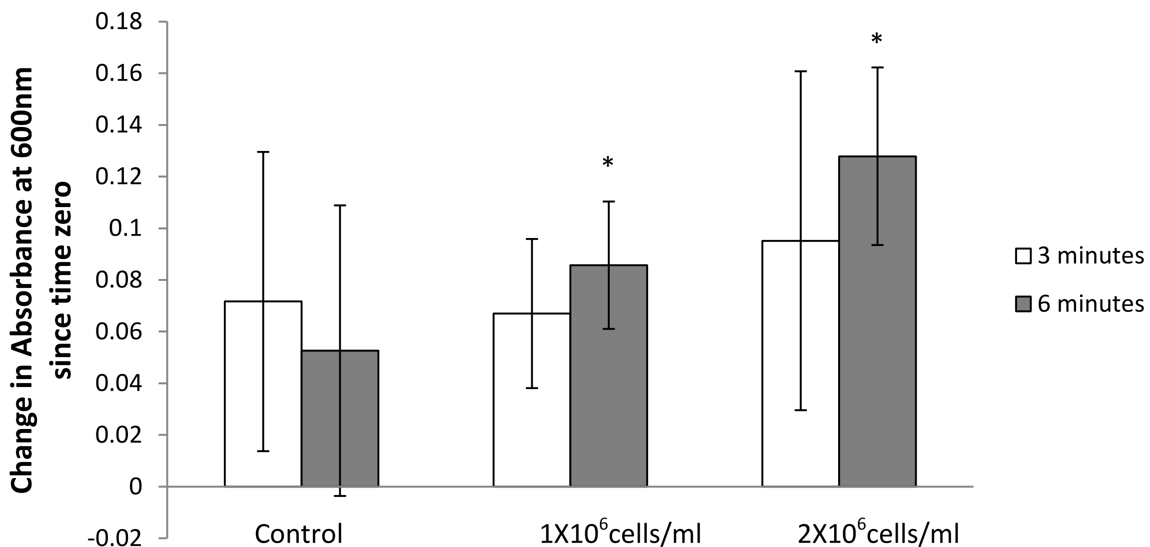


Figure 28 – Removal Of Mg^{2+} Ions From The Reaction Buffer Decreases The Rate Of Ecto-ATPase Activity. Addition of ATP (0.5mM) with MgCl left out of the reaction buffer causes a significant difference in absorbance of light at a wavelength of 600nm between samples taken from the wells containing cells at time zero and the same wells at six minutes. No significant difference was observed in the absorbance of light between samples taken from wells at time zero and three minutes in any of the wells. Data points taken at 0,3 and 6 minutes. The error bars represent standard error of the mean of each data point, N=6

A paired T-Test found a statistically significant difference in the absorption of light at 600nm six minutes after the addition of ATP (Figure 28) in the cells containing wells. No such significant difference was found at three minutes, implying that the absence of Mg^{2+} ions prevents this change in absorbance from happening as quickly as it did in the initial experiments from section 4.3.1. As change in absorbance is linked to phosphate release and it has been hypothesised that this phosphate release is a result of ATPase activity, these results would imply that ATPase activity is partially, but not completely, inhibited in the absence of Mg^{2+} ions.

It is important to note, however, that at six minutes no significant difference was observed between the change in absorbance in samples taken from wells containing cells and the control wells. This seems unusual and could be explained by the very large error bars found in this experiment. This is in turn probably a result of the alterations made to the phosphate liberation buffer. More experiments

would need to be carried out before it could be determined if this partial inhibition exists or if the results seen here are an artefact of the experimental procedure.

4.4 – The Effect Of Suramin On *Dictyostelium* Development In Submerged Culture

Suramin was capable of inhibiting the increase in phosphate ion concentration which had been observed when 0.5mM ATP was added to *Dictyostelium discoideum*. One possible explanation suggested for this was that it was inhibiting ecto-ATPase activity. It had already been implied in Chapter 3 that increasing the rate of extracellular ATP breakdown, through addition of apyrase, causes a delay in the initiation of streaming. It was, therefore, speculated that if suramin did inhibit *Dictyostelium* ecto-ATPases then it would possibly also have an effect on the timing of stream formation as it would lead to a decrease in the rate of extracellular ATP breakdown.

In order to investigate this, Submerged Culture experiments were set up as in Chapter 3, but instead of adding apyrase, 250μM suramin was added.

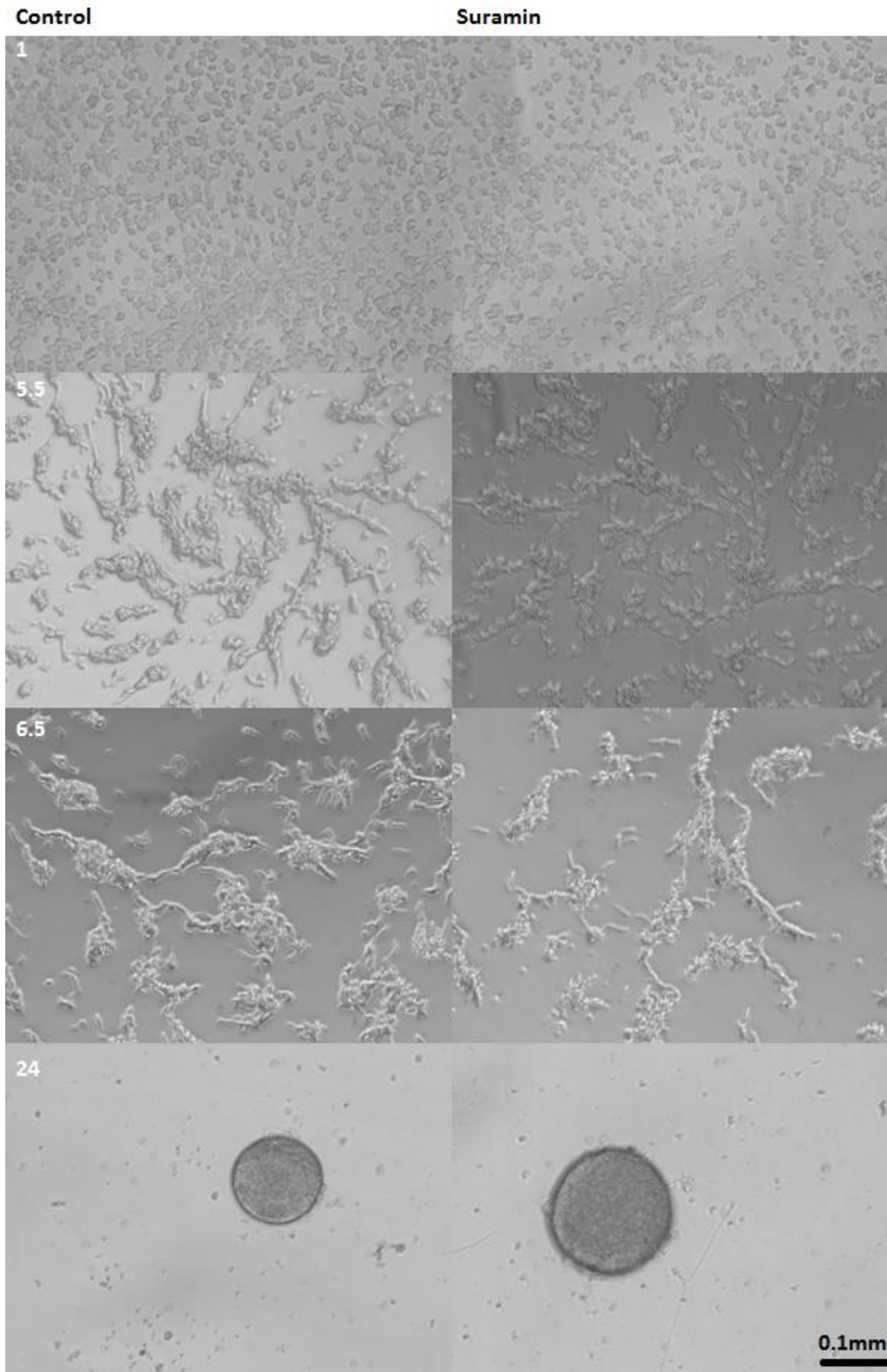


Figure 29 – No Effect Of Suramin On Development. Development of AX4 cells at a density of $5x10^5$cells/ml in cultures treated with 250µM of suramin or an equivalent volume of water over a period of 24 hours. Cells start to form early streams at 5.5 hours and by 6.5 hours streaming is fully underway. By 24 hours aggregates have formed. Time is given in hours on the left of the image. Images are of the most developmentally advanced cells found within the well.

The addition of 250µM suramin had no observable effect on the cells' development (Figure 29). As in the apyrase experiments the amount of aggregates formed in each well containing suramin treated cells was expressed as a percentage of the number of aggregates formed in the paired control well. There was no difference in the number of aggregates formed by cells developing in the presence of suramin (Figure 30). In submerged culture no difference could be detected between control and suramin treated cells.

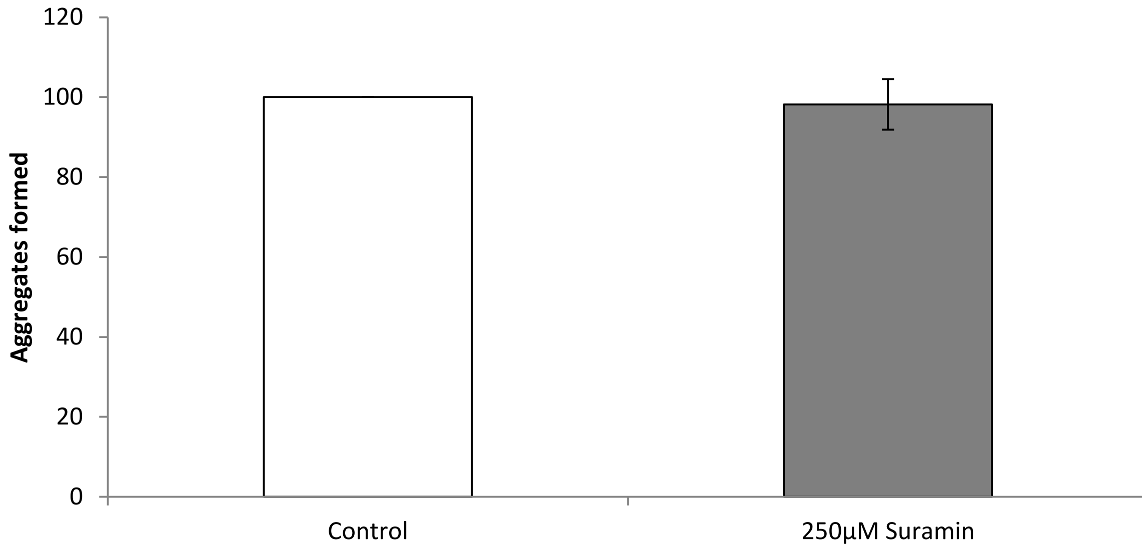


Figure 30 – Treatment With Suramin Caused No Significant Difference In The Number Of Aggregates Formed By *Dictyostelium*. Number of aggregates present in wells containing control or suramin (250µM) treated *Dictyostelium* seeded at $5X10^5$cells/ml following completion of development. The error bar represents standard error of the mean of the data, N=3, 2 wells/experiment

4.5 – The Effect Of Suramin On *Dictyostelium* Development On Agar

The effect of 250µM suramin upon development on agar was also investigated. Even though no change in timing had been observed in submerged culture, the apyrase experiments had indicated that the ATP signalling system is involved in more than just changing the timing of development.

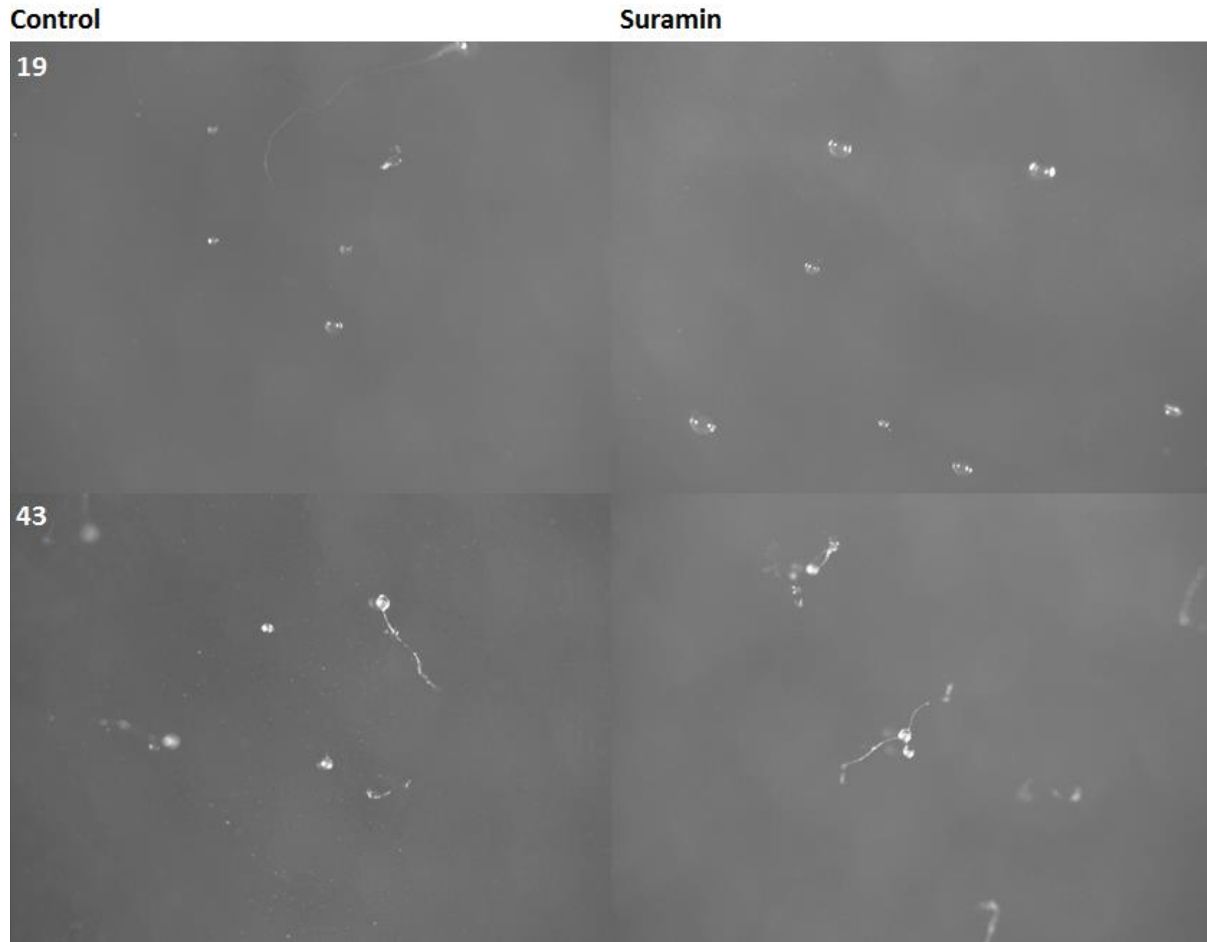


Figure 31 – Suramin Has No Effect On The Timing Of *Dictyostelium Discoideum* Development On Agar. Development of AX4 cells seeded at a density of $1x10^4$ cells/cm2 on solid non nutrient agar treated with 250µM of suramin or an equivalent volume of molecular-grade water at 19 and 43 hours after addition to the plate. At 19 hours cells in all the wells had formed mounds. By 43 hours all the plates contained fruiting bodies.

There was no difference observed in the timing of the developmental stages between either the control and suramin treated cells (Figure 31). Furthermore, there was no significant difference in the number of fruiting bodies present in the control wells and those containing cells treated with suramin (Figure 32). When the fruiting bodies were spread on bacterial lawns in order to ascertain the number of viable cells within them, however, it was found that there was a significant difference ($p<0.05$). The fruiting bodies formed from the suramin treated cells produced on average 180% as many colonies (Figure 33).

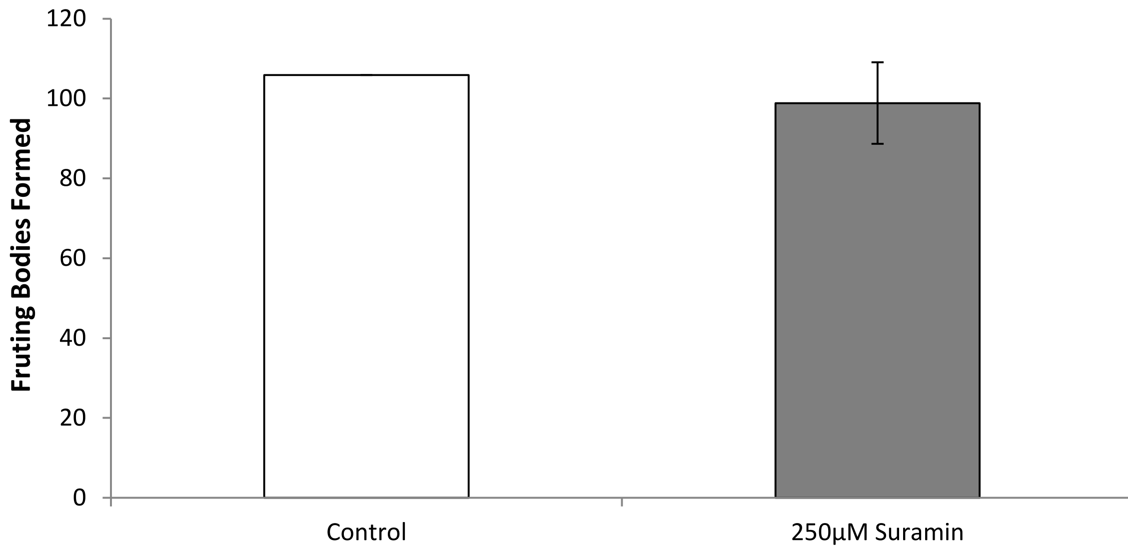


Figure 32 - Suramin Has No Effect On The Number Of Fruiting Bodies Formed. Mean number of fruiting bodies formed by *Dictyostelium* seeded at a density of $1x10^4$ cells/cm^2 on solid non nutrient agar with or without suramin (250µM). Each data point is expressed as a percentage of the number of fruiting bodies in the paired control well. The error bar represents standard error of the mean the data set, N=7, two wells/experiment.

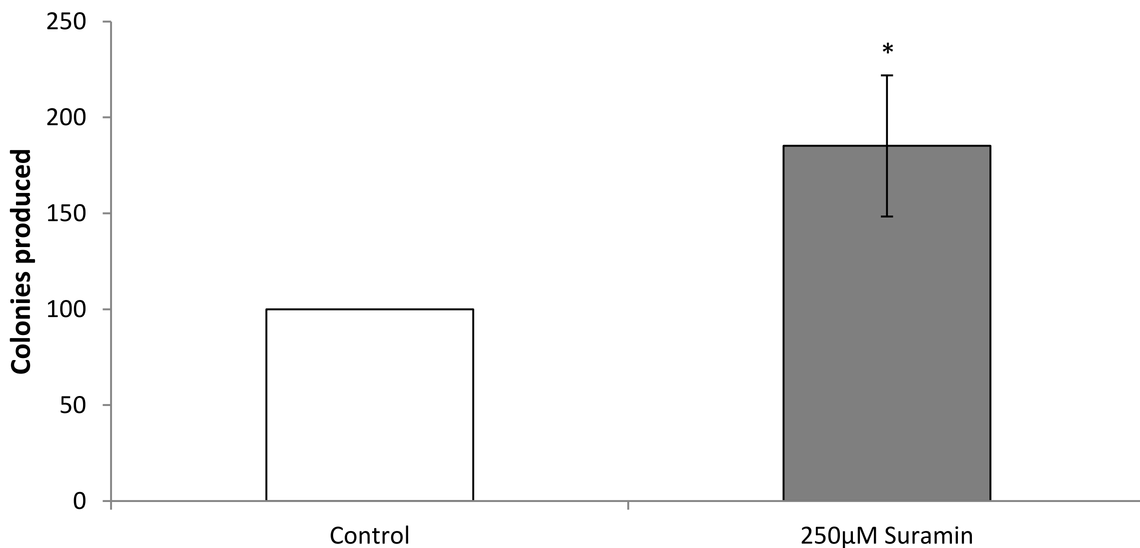


Figure 33 - Evidence For Increased Number Of Viable Cells In Fruiting Bodies Developed In The Presence Of Suramin. Number of colonies produced by 0.5ml of the mixture of a fruiting body head and 4ml of *Klebsiella* when grown upon an agar plates. Spores produced from *Dictyostelium* seeded at a density of $1x10^4$ cells/cm^2 on solid non nutrient agar with or without suramin (250µM). Each data point is expressed as a percentage of the number of colonies produced by the relevant control. N=29 plates of control and 21 of suramin. The error bar represents standard error of the mean the data set.

4.6 - Conclusions

This chapter presents evidence for the existence of endogenous ecto-ATPases in *Dictyostelium discoideum* and attempts to ascertain what role they play in development. When 0.5mM ATP is added to cells the local phosphate ion concentration increases. It is believed that this is a result of cell surface ecto-ATPases breaking down this ATP. This experiment cannot, however, rule out the possibility that cells are releasing extracellular phosphate ions in response to the ATP. It can also not be shown that ATP breakdown is carried out by ecto-ATPases and not ATPases secreted by the cells into the medium. It is important to remember, however, that the presence of these ecto-ATPases had already been shown by Parish and Weibel in 1980 and that in 2008 Ludlow used the same method used here to measure ecto-ATPase activity without mention as to a possible other source of this phosphate. Furthermore, the fact that suramin, a common inhibitor of ecto-ATPases, prevents this observed increase in phosphate concentration and alters the number of viable cells in fruiting bodies, which Chapter 3 suggests is influenced by ATP concentration, provides valuable indirect evidence that it is ecto-ATPase activity that is being observed in these experiments.

Six minutes after addition of the exogenous ATP there is no longer a significant difference between the absorbance of samples from the wells containing cells and those that do not. This would imply that the cells are absorbing the phosphate that is produced. Phosphate plays an important role in a variety of *Dictyostelium* cell functions (Zhang et al., 2005) and it seems feasible that the cells would not waste this source of it. It is unfortunate that the lack of a phosphate concentration calibration curve makes it difficult to determine whether it is indeed a significant amount of phosphate that is produced and taken up.

Mammalian ecto-ATPases require the presence of Mg^{2+} ions to operate, with an optimum concentration between 1-5mM. In fact, removal of Mg^{2+} ions by means of EGTA normally completely inhibits ecto-ATPase activity (Kukulski et al., 2005). Figure 28 suggests that this might not be the case for the *Dictyostelium* ecto-ATPases as, although phosphate ion concentration decreases in the buffer without Mg^{2+} ions, it is not completely stopped. It is important to note, however, that while the buffer was made up without Mg^{2+}, no attempts were made to keep it out of the extracellular medium and EGTA was not added. It is already known that the other cation necessary for ecto-ATPase activity in mammals, Ca^{2+} (Kukulski et al., 2005), is released extracellular by *Dictyostelium discoideum* (Sivaramakrishnan and Fountain, 2012). It seems possible that *Dictyostelium* could also release Mg^{2+} into the medium, the lower rate of activity being a result of the lower concentration caused by the cells being the only source of Mg^{2+}. Indeed, it is known that the cells release their Ca^{2+} ions in response to ATP and it seems plausible that one of the reasons is to increase the activity of the ecto-ATPases which break down this ATP.

There are two families of cell surface proteins which break down ATP. Ecto-ATPases break down ATP in preference to ADP, whereas the closely related ecto-ATP diphosphohydrolases break down both ATP and ADP equally (Zimmermann et al., 1998). The protein observed by Parish and Weibel in 1980 appeared to break down ATP over ADP implying that it was only an ecto-ATPase. The phosphate liberation experiments above do not differentiate between the two and, in fact, the apyrase used in the development experiments was of the type that would break down both nucleotides equally. The phenomenon observed did, however, react to suramin and Mg^{+} in the same way as the one observed by Parish and Weibel, again implying that the experiment is measuring the ecto-ATPase's activity.

As stated earlier, there are no specific inhibitors of ecto-ATPases (Knowles, 2011), so there is a worry that the results seen from the introduction of suramin and removal of Mg^{+} represent responses of other cellular pathways rather than inhibition of the ecto-ATPases. In both treatments, however, there was a lack of significant change in phosphate concentration during the first three minutes, which implies that the same pathways are being affected in both. This is likely to be the ecto-ATPase as it is unlikely that both treatments would have similar unknown side effects to a hypothesised effect.

One of the known side effects of suramin is that it can inhibit P2X receptors and perhaps one might have expected a similar reaction in the suramin treated cells as in those treated with apyrase. Apyrase is, after all, assumed to cause its effect by preventing cells from detecting ATP and ADP, removing it before it can activate receptors. This is not the case, however, and indeed the existing work on *Dictyostelium discoideum's* responses to nucleotides indicate that, although its method of ATP/ADP detection is similar to P2X in many ways, it is not suramin sensitive (Ludlow et al., 2008).

Given that the presence of exogenous ATP was demonstrated in chapter 3, it would be reasonable to assume that inhibition of the cell's natural ecto-ATPases by suramin would raise the extracellular ATP levels. The major obstacle preventing the experimental confirmation of this is that suramin inhibits luciferase (Tatur et al., 2007) and so the assay used throughout this project to measure the presence of ATP would be unsuited for such experiments. Without this one cannot conclusively state whether the ATP levels do indeed rise in the presence of suramin or whether suramin actually impacts ATP breakdown in development buffer. The fact that one observes an alteration in the number of viable cells present within a fruiting body indicates that adding suramin during development causes interference with a system which Chapter 3 had suggested is regulated by ATP.

Chapter 3 suggests that removal of exogenous ATP by the ATPase apyrase causes a delay in stream formation, but these results imply that inhibiting the breakdown of this ATP has no effect on the timing. This indicates that there is not a direct correlation between rate of ATP concentration and the initiation of streaming. One possible explanation could be that there is an ATP threshold that must be met for normal streaming to occur. If the ATP concentration falls below this level (for example, due to addition of apyrase) then the timing of streaming is impaired. The cells are indifferent, however, to the concentration either above or below this level. This accords with established knowledge of Purinergic receptors in other organisms where it has been shown that the ATP concentration must be a certain level for cation influx to occur (Baricordi et al., 1996). While it is important to remember that the ATP response of *Dictyostelium discoideum* does not correspond to any currently known purinergic receptor, it seems logical that it would have many of the same features.

An alternative explanation would be that, although the response to ATP is concentration dependent, under control conditions the amount of ATP released gives the maximal response and the cells are physically incapable of developing any faster. Below the concentration found in control conditions, however, the rate of stream formation may be proportional to ATP concentration. Experiments into adjusting the concentration of apyrase added to the cells and comparing the timing between these would be a useful way of determining which hypothesis is more likely.

That fruiting bodies formed from the suramin treated cells were able to produce more colonies than the control ones, despite there being no difference in the number of fruiting bodies themselves, lends credence to one theory postulated in the previous chapter. Interfering with the ATP signalling system, rather than changing the number of cells present in the fruiting bodies, could instead alter the number of these cells that are viable. It may be that the cell number remains the same within these fruiting bodies, but the number detectable during the bacterial lawn experiments changes. This hypothesis has

the advantage of not raising the question of where the additional cells within the fruiting bodies treated from suramin came from, as they would also be present under control conditions. One should bear in mind that, unlike in the apyrase experiments, suramin did not appear to alter the number of unaggregated cells following development. This would imply that a similar percentage of the cells within each well have entered the fruiting bodies in those containing cells treated with suramin as those under control conditions.

This hypothesis does, however, raise the question of what unviable cells would be doing within the fruiting bodies under control conditions. Unfortunately the fact that unviable cells are by their very nature unviable, makes it difficult to test for their presence short of cracking open the fruiting bodies and physically counting the cells. This is unfeasible. Some of the experiments in this project produced fruiting bodies containing as few as 100 viable cells: an experiment where 25 colonies formed from 0.5ml of KA/Spore mix had a mean of 200 cells in the 4ml of KA into which the two spores had been initially suspended. The haemocytometer used in this lab loses accuracy when counting fewer than $5x10^5$ cells/ml and has to be loaded with 10µl for each reading. Even assuming that one did not make more volume than required, one would still need to use 50 fruiting bodies for each count, even without taking into account the need for repeats and observing the cells under the different treatments.

Chapter 5 - Bioinformatics

5.1 – Aim

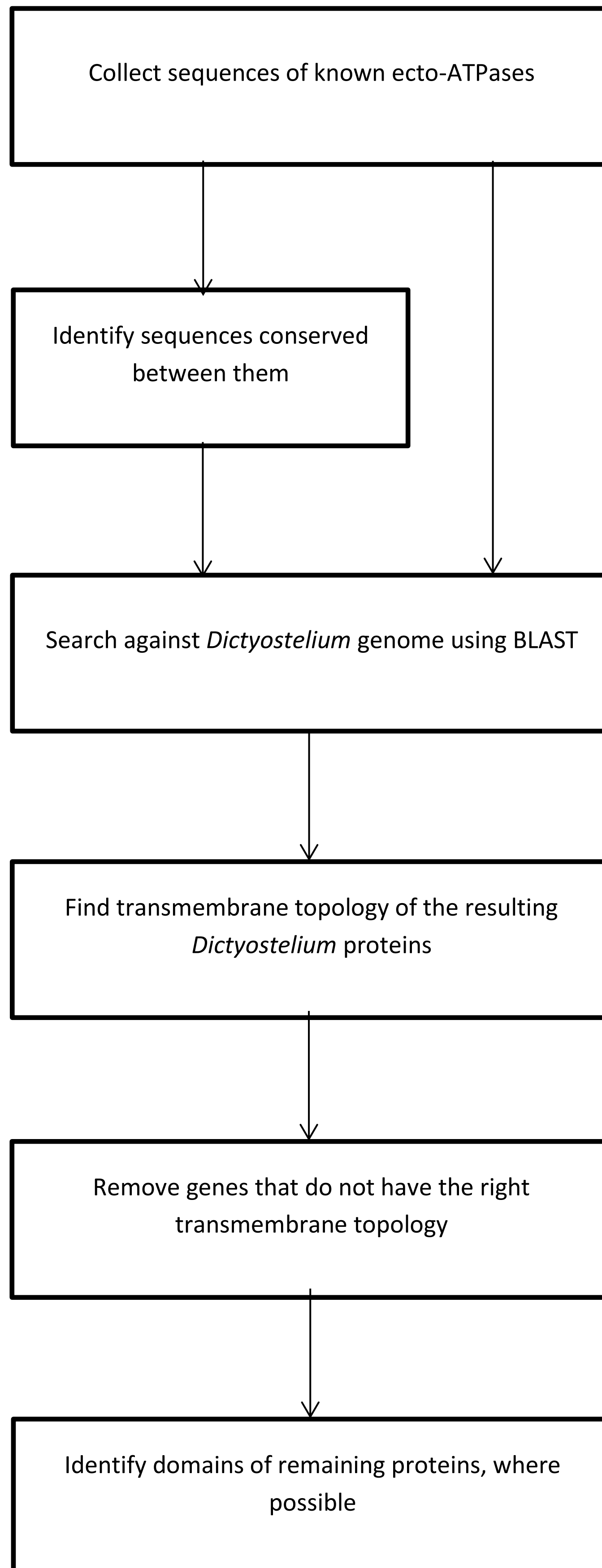
This chapter outlines the bioinformatic techniques used in an attempt to discover the genes responsible for the ATPase activity observed in *Dictyostelium discoideum*.

5.2 – Introduction

The experiments carried out throughout this thesis imply that extracellular ATP is used by *Dictyostelium discoideum* cells to coordinate their development. Knowledge of the genes involved would allow the creation of mutants, which could be better used to explain how this ATP signalling system works. The initial discovery of *Dictyostelium* P2X receptors was made using bioinformatic techniques (Fountain et al., 2007). One would assume that if the ATP receptor which the cells were using in these experiments was easily detectable by bioinformatics assays then it would have been identified at this point.

The cells, however, have been confirmed to possess ecto-ATPases (Parish and Weibel, 1980), and it is suggested in Chapter 4 that inhibition of these interferes with this ATP signalling system, as does adding extra endogenous ATPases. Although detection of this ATPase would not directly reveal the receptor, it would allow further investigation into modulating the pathway responses.

5.3 – Overview Of The Process Used



5.4 – Conserved Sequences

The amino acid sequences of the known Ecto-ATPases found in various other species were gathered from the PubMed database. For convenience in cataloguing them, each gene was referred to by its Gene Index, or gi, number.

Arabidopsis thaliana-	gi|339283644 - nucleoside triPhosphate diphosphohydrolase 3
	gi|339283646 - nucleoside triPhosphate diphosphohydrolase 4
	gi|339283648 - nucleoside triPhosphate diphosphohydrolase 5
	gi|339283650 - nucleoside triPhosphate diphosphohydrolase 6
	gi|339283652 - nucleoside triPhosphate diphosphohydrolase 7
Bos taurus -	gi|27806971 - ectonucleoside triPhosphate diphosphohydrolase 1
	gi|296472657 - ectonucleoside triPhosphate diphosphohydrolase 1
	gi|300795252 - ectonucleoside triPhosphate diphosphohydrolase 5
	gi|118150950 - ectonucleoside triPhosphate diphosphohydrolase 8
	gi|296481999 - ectonucleoside triPhosphate diphosphohydrolase 8
Danio rerio -	gi|57525937 - ectonucleoside triPhosphate diphosphohydrolase 1
	gi|54261809 - ectonucleoside triPhosphate diphosphohydrolase 2
	gi|134133300 - ectonucleoside triPhosphate diphosphohydrolase 3
	gi|50539906 - ectonucleoside triPhosphate diphosphohydrolase 4
	gi|62955697 - ectonucleoside triPhosphate diphosphohydrolase 6
Drosophila melanogaster -	gi|194369341- NTPDase 6
Gallus gallus -	gi|16518970 - ecto-ATP-diphosphohydrolase
	gi|45384424 - ectonucleoside triPhosphate diphosphohydrolase 2
	gi|45383632 - ectonucleoside triPhosphate diphosphohydrolase 8
Homo sapiens -	gi|45580700 - ectonucleoside triPhosphate diphosphohydrolase 1
	gi|45827718 - ectonucleoside triPhosphate diphosphohydrolase 2
	gi|166197702 - ectonucleoside triPhosphate diphosphohydrolase 3
	gi|166235893 - ectonucleoside triPhosphate diphosphohydrolase 6
	gi|9966821- ectonucleoside triPhosphate diphosphohydrolase 7
	gi|110431368 - ectonucleoside triPhosphate diphosphohydrolase 8
	gi|14547943 - Ectonucleoside triPhosphate diphosphohydrolase 8
	gi|145314002 - small cell lung carcinoma ecto-ATPase
	gi|59003436 - liver ecto-ATP-diphosphohydrolase

	gi|358440048 - Crystal Structure & Domain Rotation Ntpdase1 Cd39
Leishmania major -	gi|45120584 - putative NTPDase
Lolium perenne -	gi|170791250 – Apyrase
Mus musculus -	gi|6753346 - ectonucleoside triPhosphate diphosphohydrolase 1
	gi|161484610 - ectonucleoside triPhosphate diphosphohydrolase 2
	gi|18093090 - ectonucleoside triPhosphate diphosphohydrolase 4
	gi|71061460 - ectonucleoside triPhosphate diphosphohydrolase 5
	gi|82617566 - ectonucleoside triPhosphate diphosphohydrolase 7
	gi|112821698 - ectonucleoside triPhosphate diphosphohydrolase 8
Pongo abelii -	gi|207080274 - ectonucleoside triPhosphate diphosphohydrolase 7
Rattus norvegicus -	gi|12018242 - ectonucleoside triPhosphate diphosphohydrolase 1
	gi|30017439 - ectonucleoside triPhosphate diphosphohydrolase 3
	gi|1675825 - ectonucleoside triPhosphate diphosphohydrolase 6
	gi|75677557 - ectonucleoside triPhosphate diphosphohydrolase 8
Salmo salar -	gi|223649432 - Ectonucleoside triPhosphate diphosphohydrolase 2
	gi|209154216 - Ectonucleoside triPhosphate diphosphohydrolase 2
	gi|209147767 - Ectonucleoside triPhosphate diphosphohydrolase 6
Sus scrofa -	gi|47523398 - ectonucleoside triPhosphate diphosphohydrolase 1
Trifolium repens -	gi|170791248 – Apyrase
	gi|170791246 – Apyrase
Trypanosoma brucei -	gi|45120586 - TPA_exp: c
Xenopus laevis -	gi|74039691 - ecto-nucleosidase triPhosphate diphosphohydrolase 3
	gi|134085336 - ectonucleoside triPhosphate diphosphohydrolase 4
	gi|148232431 - ectonucleoside triPhosphate diphosphohydrolase 6
	gi|74039689 - ecto-nucleosidase triPhosphate diphosphohydrolase 6
	gi|62859996 - ectonucleoside triPhosphate diphosphohydrolase 7
Xenopus (Silurana) tropicalis -	gi|74231263 - NTPDase5
	gi|74231265 - NTPDase6

Having gathered the various sequences, the next step was to find regions that were highly conserved between them. The reasoning being that if the gene that fulfils a function has the same sequence of amino acids, even in species that are only very distantly related, then this region is likely to be highly important for the carrying out of this function (Alberts, 2008).

One cannot, however, just line up two proteins next to each other in order to find these conserved sequences. The millennia over which the genes have evolutionarily diverged are more than enough time for multiple amino acid insertions, inversions and deletions to occur, making the distance between these conserved regions vary widely between organisms. In order to find these conserved regions, one must carry out a "sequence alignment." A computer program is used to compare many sequences and line them up on the basis of the incidence of certain amino acid sequences at certain positions along the protein. One such program is ClustalW (Thompson et al., 1994).

The amino acid sequences of the proteins, which had been identified by PubMed, were entered into ClustalW and the conserved regions identified. This output is displayed over the next two pages in Figure 34.

Sequence	1	2	3
Bos|gi|300795252|ref|NP_001179	LYGIMFDAGSTGTRIHVY	VLKATAGLRLL	IMDGSYEGILAWVTVN
Mus|gi|71061460|ref|NP_031673.	FYGIMFDAGSTGTRIHVY	VLKATAGLRLL	IMDGSYEGILAWVTVN
Xenopus|gi|74231263|gb|ABA0073	IYGIMFDAGSTGTRIHIY	VLKATAGLRLL	IINGADEGIFAWITVN
Danio|gi|62955697|ref|NP_00101	QYGIMIDAGSTGTRIHIF	MLRATAGLRLL	IMDGTDEGMSAWITIN
Salmo|gi|209147767|gb|ACI32905	QYGVMFDAGSTGTRIHVF	VLKATAGLRLL	IMDGTNEGISAWITVN
Homo|gi|166235893|ref|NP_00123	FYGIMFDAGSTGTRVHVF	VLKATAGLRLL	IMNGTDEGVSAWITIN
Rattus|gi|16758256|ref|NP_4459	FYGIMFDAGSTGTRIHVF	VLKATAGLRLL	IMNGTDEGVSAWITVN
Xenopus|gi|148232431|ref|NP_00	FYGIMFDAGSTGTRIHIY	VLKATAGLRLL	IMNGTDEGIFAWITVN
Xenopus|gi|74039689|gb|AAZ9487	FYGIMFDAGSTGTRIHIY	VLKATAGLRLL	IMNGTDEGIFAWITVN
Xenopus|gi|74231265|gb|ABA0073	FYGIMFDAGSTGTRIHVY	VLKATAGLRLL	IMNGTDEGIFAWITVN
Drosophila|gi|194369341|gb|ACF	QYAAIIDAGSTGSRVLAY	VLKATAGLRLL	IMDGTDEGIFSWFTVN
Trifolium|gi|170791248|gb|ACB3	SYAVVFDAGSTGSRVHVY	ALGATAGLRLL	IIDGTQEGSYLWVTIN
Trifolium|gi|170791246|gb|ACB3	SYAVIFDAGSTGSRVHVY	SLGATAGLRLL	IIDGTKEGSYLWVAIN
Lolium|gi|170791250|gb|ACB3828	KYAVIFDAGSTGTRVHVF	KLGATAGLRII	VLEGSQEGSYLWVALN
Arabidopsis|gi|339283646|gb|AE	HYSVIIDGGSSGTRVHVF	RLMATAGMRLL	VISGSDEGVYAWVVAN
Arabidopsis|gi|339283648|gb|AE	HYSVIIDAGSSGTRIHVF	RLMATAGMRLL	VISGTDEGIYAWVVAN
Arabidopsis|gi|339283644|gb|AE	RYSVLIDAGSSGTRVHVF	RLMATAGMRLL	VISGSDEGIYSWITAN
Arabidopsis|gi|339283650|gb|AE	RYSVVIDGGSTGTRIHVF	RLMATAGMRLL	VISGSDEGVYAWVVAN
Bos|gi|118150950|ref|NP_001071	KFGIVFDAGSSHTSLFVY	VLGATAGMRLL	LLTGQDEGAFGWITIN
Bos|gi|296481999|gb|DAA24114.1	KFGIVFDAGSSHTSLFVY	VLGATAGMRLL	LLTGQDEGAFGWITIN
Homo|gi|110431368|ref|NP_00102	KFGIVFDAGSSHTSLFLY	VLGATAGMRLL	LLAGQAEGAFGWITVN
Homo|gi|59003436|gb|AAW83516.1	KFGIVFDAGSSHTSLFLY	VLGATAGMRLL	LLAGQAEGAFGWITVN
Mus|gi|112821698|ref|NP_082369	KFGIVFDAGSSHTSLFVY	VLGSTAGMRLL	ILAGQDEGAFGWITIN
Rattus|gi|75677557|ref|NP_0010	KFGILFDAGSSHTSLFVY	VLGATAGMRLL	ILAGQDEGAFGWITVN
Gallus|gi|45383632|ref|NP_9895	KYGLVFDAGSTHTALYVY	VLGATAGMRLL	ILTGNEEGSFGWITVN
gi|14547943|sp|O93295.1|ENTP8_	KYGLVFDAGSTHTALYVY	VLGATAGMRLL	ILTGNEEGSFGWITVN
Gallus|gi|16518970|gb|AAL25086	KYGLVFDAGSTHTALYVY	VLGATAGMRLL	ILTGNEEGSFGWITVN
Salmo|gi|223649432|gb|ACN11474	MYGVVLDAGSSHTAMYIY	VLGATAGMRLL	ILSGQEEGAYGWVTVN
Salmo|gi|209154216|gb|ACI33340	MYGIVLDAGSSHTAMYIY	VLGATAGMRLL	ILSGQEEGAYGWVTVN
Danio|gi|54261809|ref|NP_00100	MYGIVLDAGSSHTSMFIY	VLGATAGMRLL	ILTGQEEGAYGWITVN
Homo|gi|45827718|ref|NP_982293	KYGIVLDAGSSHTSMFIY	VLGATAGMRLL	ILSGQEEGVFGWVTAN
Homo|gi|145314002|gb|ABP58644.	KYGIVLDAGSSHTSMFIY	VLGATAGMRLL	ILSGQEEGVFGWVTAN
Mus|gi|161484610|ref|NP_033979	KYGIVLDAGSSHTSMFVY	VLGATAGMRLL	ILSGQDEGVFGWVTAN
Rattus|gi|75677557|ref|NP_0010	KFGILFDAGSSHTSLFVY	VLGATAGMRLL	ILAGQDEGAFGWITVN
Gallus|gi|45383632|ref|NP_9895	KYGLVFDAGSTHTALYVY	VLGATAGMRLL	ILTGNEEGSFGWITVN
gi|14547943|sp|O93295.1|ENTP8_	KYGLVFDAGSTHTALYVY	VLGATAGMRLL	ILTGNEEGSFGWITVN
Gallus|gi|16518970|gb|AAL25086	KYGLVFDAGSTHTALYVY	VLGATAGMRLL	ILTGNEEGSFGWITVN
Salmo|gi|223649432|gb|ACN11474	MYGVVLDAGSSHTAMYIY	VLGATAGMRLL	ILSGQEEGAYGWVTVN
Salmo|gi|209154216|gb|ACI33340	MYGIVLDAGSSHTAMYIY	VLGATAGMRLL	ILSGQEEGAYGWVTVN
Danio|gi|54261809|ref|NP_00100	MYGIVLDAGSSHTSMFIY	VLGATAGMRLL	ILTGQEEGAYGWITVN
Homo|gi|45827718|ref|NP_982293	KYGIVLDAGSSHTSMFIY	VLGATAGMRLL	ILSGQEEGVFGWVTAN
Homo|gi|145314002|gb|ABP58644.	KYGIVLDAGSSHTSMFIY	VLGATAGMRLL	ILSGQEEGVFGWVTAN
Mus|gi|161484610|ref|NP_033979	KYGIVLDAGSSHTSMFVY	VLGATAGMRLL	ILSGQDEGVFGWVTAN
Gallus|gi|45384424|ref|NP_9902	KYGIVLDAGSSHTAVFIY	VLGATAGMRLL	ILSGEEEGVFGWITAN
Bos|gi|27806971|ref|NP_776961.	KFGIVLDAGSSHTSLYIY	VLGATAGMRLL	IISGQEEGAYGWITVN
Bos|gi|296472657|gb|DAA14772.1	KFGIVLDAGSSHTSLYIY	VLGATAGMRLL	IISGQEEGAYGWITVN
Sus|gi|47523398|ref|NP_999318.	KFGIVLDAGSSHTSLYIY	VLGATAGMRLL	IITGQEEGAYGWITIN
Cd39|gi|358440048|pdb|3ZX3|D	KYGIVLDAGSSHTNLYIY	VLGATAGMRLL	IITGQEEGAYGWITIN
Rattus|gi|12018242|ref|NP_0721	KYGIVLDAGSSHTNLYIY	VLGATAGMRLL	IITGQEEGAYGWITIN
Mus|gi|6753346|ref|NP_033978.1	KYGIVLDAGSSHTNLYIY	VLGATAGMRLL	IITGQEEGAYGWITIN
Homo|gi|45580700|ref|NP_001767	KYGIVLDAGSSHTSLYIY	VLGATAGMRLL	IITGQEEGAYGWITIN
Danio|gi|57525937|ref|NP_00100	KYGIVLDAGSSHTSVFIY	VLGATAGMRLL	ILSGQEEGAFGWITVN
Homo|gi|166197702|ref|NP_00123	KYGIVLDAGSSRTTVYVY	HLGATAGMRLL	IISGQEEGVYGWITAN
Rattus|gi|30017439|ref|NP_8352	KYGIVLDAGSSRTTVYVY	VLGATAGMRLL	IISGQEEGVYGWITAN
Xenopus|i|74039691|gb|AAZ94877	KYGIVLDAGSSRTTVYVY	VLGATAGMRLL	IISGQEEGLYGWITVN
Danio|gi|134133300|ref|NP_0010	KYGIVLDAGSSRTTVYLY	VLGATAGMRLL	IISGQEEGLYGWITVN
Mus|gi|18093090|ref|NP_080450.	NYGIVVDCGSSGSRIFVY	VILCTAGMRVL	VISGKQEGVYAWIGIN
Xenopus|gi|134085336|ref|NP_00	NYGIVVDCGSSGSRIFVY	VILCTAGLRVL	VISGKQEGVYAWIGIN
Danio|gi|50539906|ref|NP_00100	NYGLVVDCGSSGSRVFVY	VILCTAGMRVL	VISGKQEGVYAWIGIN
Homo|gi|9966821|ref|NP_065087.	NYGLVVDCGSSGSRIFVY	VILCTAGMRLL	VISGKQEGVYAWIGIN
Pongo|gi|207080274|ref|NP_0011	NYGLVVDCGSSGSRIFVY	VILCTAGMRLL	VISGKQEGVYAWIGIN
Mus|gi|82617566|ref|NP_444333.	NYGLVVDCGSSGSRIFVY	VILCTAGMRLL	VISGKQEGVYAWIGIN
Xenopus|gi|62859996|ref|NP_001	NYGVVVDCGSSGSRVFVY	VILCTAGMRIL	VISGKQEGVYAWISIN
Leishmania|gi|45120584|tpg|DAA	VYDVVIDAGSTGSRVHVF	LKATAGLRLL	IISGAXXGVXXWXTVN
Trypanosoma|gi|45120586|tpg|DA	RYSVVFDAGSTGSRVHVY	ILKATAGLRRI	ILEGREEGPLAWLTVN
Arabidopsis|gi|339283652|gb|AE	RYYVVFDCGSTGTRAYVY	FVYATAGVRRL	IISGTEEAYFGWTALN

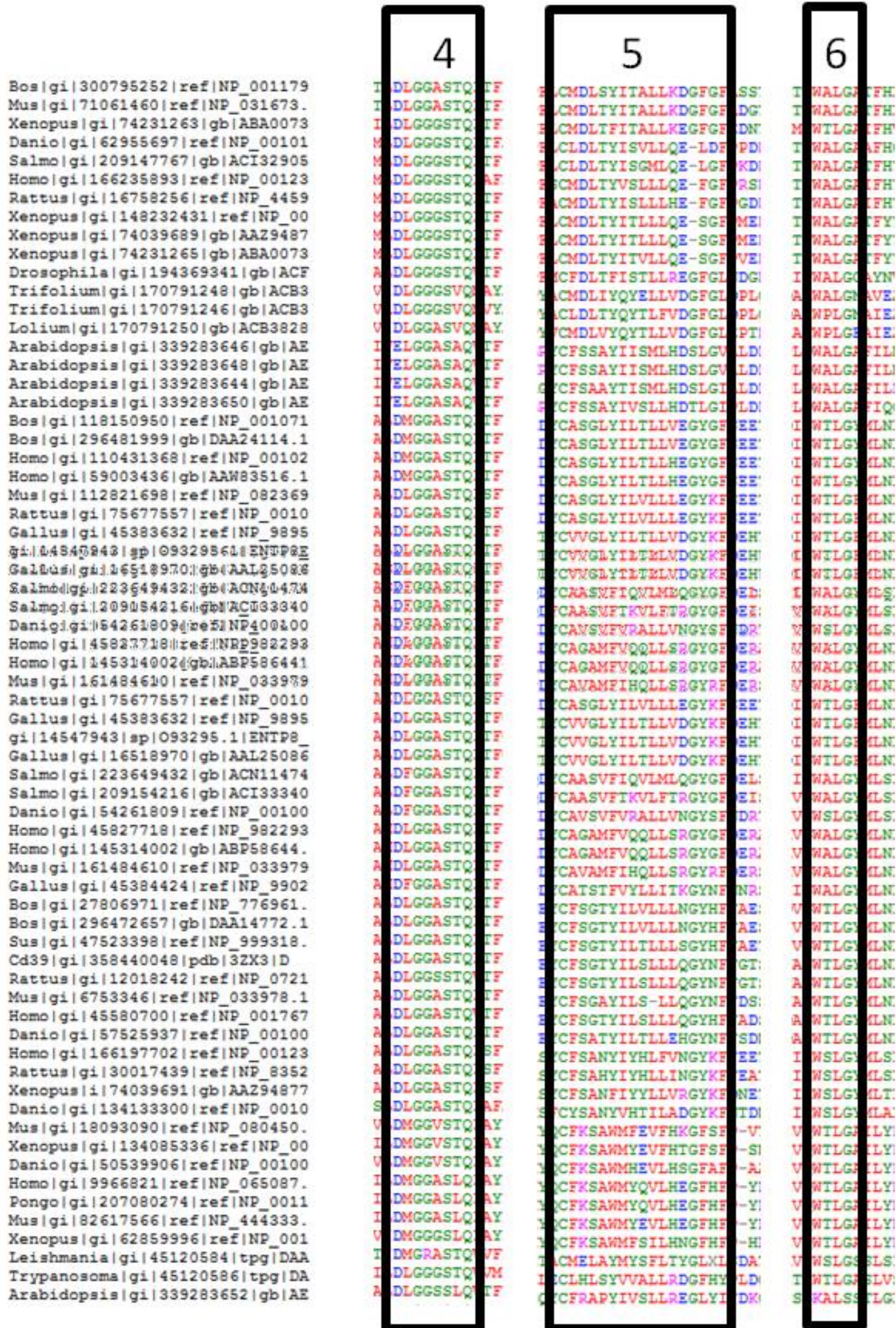


Figure 34 - Sections Of The Clustalw Output (Larkin et al., 2007) With The Regions That Were Highly Conserved Marked And Numbered

In total six conserved regions were found. The sequences are as follows, with X being used to represent a space where any amino acid could go:

1 - Y/FXXXXDG/A/CGST/SXT/SXXXXY/F

2- L/IXA/CTAGM/LRXL/I

3 - I/V/LI/L/MXGXXEGXXXWXXXN

4 - D/EL/F/MGGXSXQI/V/M

5 - CXXXXY/F/WXXXV/L/F/M

6 – WXLG

5.5 – Protein BLAST Search

Having obtained the amino acid sequences of regions that were conserved in already discovered ecto-ATPases, these were then compared to the complete list of coding amino-acid sequences identified by the *Dictyostelium discoideum* genome project in order to discover proteins that shared these regions. To do this the program "protein BLAST" was used. This compares an input sequence for similar patterns of amino acid distrubution across all known genes. Initially the sequences were run through BLAST with an expect value of ten, but not many results were produced. Subsequently the expect value was raised to 100. The expect value gives the program a measure of how many results one would expect to find by chance in a genome of that size, regardless of how relevant. By raising the value one gets more results, but with less similarity to the input sequence (ncbi.nlm.nih.gov). Another reason it was felt necessary to take this step was that during the search for the *Dictyostelium* P2X receptors, it was found that they only had very slight similarity to the genes' known counterparts in other organisms. Homologous sequences were only found in one small region of the transmembrane domain and a high expect value needed to be entered to find them (Fountain, 2012, personal communication). If known purinergic *Dictyostelium* genes had such small similarity, it sets a precedent that the others could be equally dissimilar. By casting the net wider one increased the chance of finding them.

Proteins whose function are unknown are referred to in the ncbi database as "hypothetical protein" followed by an identification number. Only a gene with unknown function would have potential to be an ecto-ATPases so only the hypothetical proteins found in the results from the BLAST search were gathered. Each conserved region was run through BLAST on its own, as well as all the segments together seperated by dashes. It was reassuring to see Sequence 6 gave known ATPases in the results (though not ecto-ATPases), implying that this was the section with the ATPase activity, while sequence 3 gave ATP binding cassettes.

Also, each of the genes gathered from the PubMed database were also run through a BLAST alignment against the *Dictyostelium* genome with the hope that, even if no gene had enough similarity to the (relatively short) conserved regions, one still might be similar enough to the same gene in a different organism to be detected by a BLAST search.

In total 3640 hypothetical proteins were identified. A table of these can be found, without the duplicates removed, on the CD included at the back of this thesis.

5.6 – Transmembrane Alignment

With so many proteins identified a way had to be found of narrowing them down to a more manageable number. It was decided to do this on the basis of the transmembrane regions. Programs exist that can use the Markov model to identify sections of an amino acid sequence that are likely to be transmembrane regions. These work on the basis of such factors as the higher density of hydrophobic residues within a transmembrane alpha helix or the increase in positively charged residues located on segments of protein within the cytoplasm (Kahsay et al., 2005). TMHMM is one such program.

Each of the hypothetical proteins gathered from the BLAST searches was run through the TMHMM program and the number of transmembrane regions predicted was recorded. The typical structure of an ATPase is two transmembrane domains with a segment of protein on the outside of the cell between them; a shape that is curiously reminiscent of that of the P2X receptor (Zimmermann et al., 1998). Only those proteins which were predicted to possess two transmembrane regions, with the section between them located on the outside of the cell, were carried forward to the next stage of the investigation. Examples of TMHMM outputs and how they were interpreted can be seen in Figure 35.

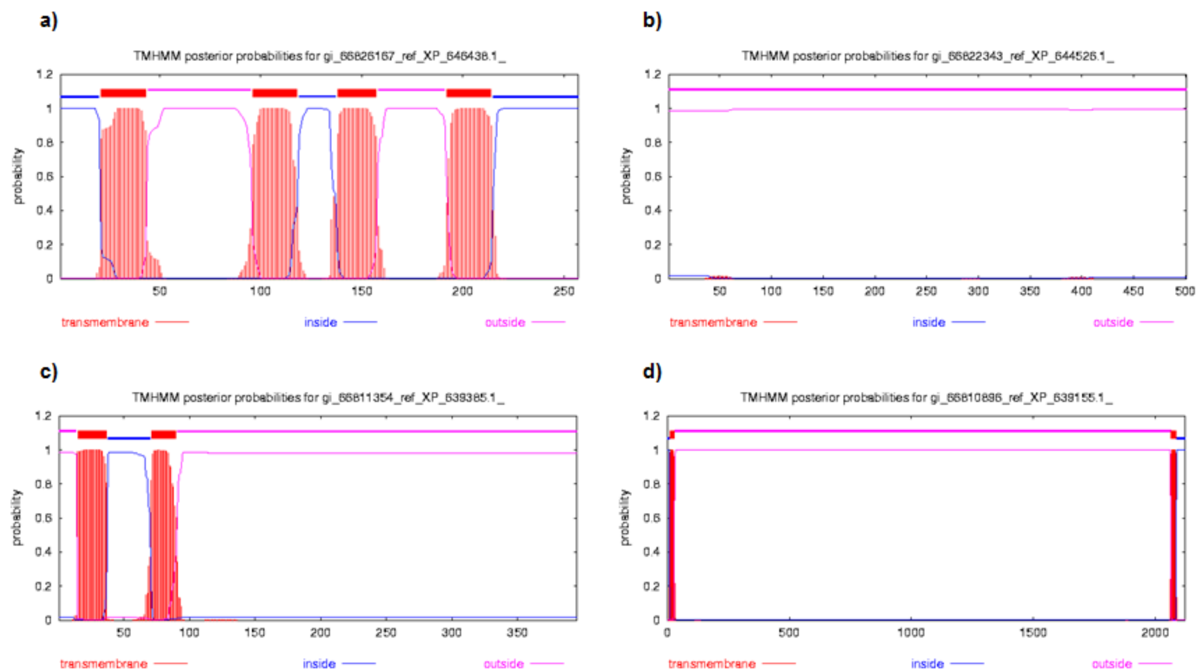


Figure 35 –TMHMM Output For Four Proteins (Analysis, 2007). a) Protein gi_66826167 has four transmembrane domains, which is too many so it is discarded. b) Protein gi_66822343 has no transmembrane domains. It cannot be anchored in the membrane and so it is discarded. c) Protein gi_66822343 contains two transmembrane regions, but the segment between them is located on the inside of the cell membrane. Even if this protein has ATPase activity, it would be acting on nucleotides on the inside of the cell and so is irrelevant to this investigation. It is discarded. d) Protein gi_66810896 has two transmembrane domains separated by an external region. This is carried on for further investigation.

Although the majority of ecto-ATPases contain two transmembrane regions, it is true that some are known to have only one. If the *Dictyostelium* ecto-ATPases are like this, then they would be falsely discounted by this investigation. The number of proteins, however, had to be narrowed down and these single transmembrane ecto-ATPases have so far only been found to break down internal ATP (Knowles, 2011), while Chapter 4 suggest that the protein looked for here acts on extracellular ATP.

The external segment of the protein is a vital component of an ecto-ATPases. The ATPases operate on ATP on the outside of the cell membrane and, without having a region on the outside, it is impossible for the catalytic domain to contact the substrate.

Once each protein's transmembrane alignment had been predicted, the results were added to the list found on the CD at the back of this thesis. The proteins that fit the criteria were added to a new list, found in this thesis's Appendix, and now that the number of proteins was smaller, the duplicate entries were merged. Finally 69 proteins remained.

5.7 – Identifying Domains

There had been identified 69 *Dictyostelium discoideum* proteins that were similar enough to ecto-ATPases to be returned from the BLAST search and were predicted to have a transmembrane conformation that corresponded to the most common ecto-ATPase shape. At this point there were no more large scale screening techniques that could be applied to all potential proteins and they had to be examined individually. Thankfully *Dictyostelium discoideum*, as mentioned in the introduction, was one of the first genomes to be sequenced and as a result has been the subject of extensive work. The Dictybase website consolidates many useful resources in one place. Most other organisms lack such a useful centralised source of information.

The database has an entry for every gene identified by the *Dictyostelium* Genome Project, both characterised and hypothetical. Each entry contains a link to the InParanoid entry for the gene, if it exists, and a link to the Dictyexpress entry which contains substantial amount of data on the expression of the gene under different conditions. The Protein Information tab contains graphical interfaces. These highlight regions of the protein sequence which correspond to known families of protein domains available in the InterPro and Panther protein databases, identified via the UniProt program (Gaudet et al., 2011). From Dictybase one could easily access large amounts of information on each protein to try and evaluate whether it had the potential to be an ecto-ATPase. The Panther database lists nucleotide phosphatase domains as being part of the PTHR11782 family and this in particular was sought in the outputs.

InParanoid is a program that attempts to find orthologues of a gene within other organisms under the assumption that the same gene in different organisms will have the same function. In particular it attempts to differentiate genes that have been duplicated and diverged before a speciation event as opposed to those that did so afterwards. The most recent version as of the time of writing, version 7.0, compares the selected protein to 100 different species with sequenced genomes, which between them contain 1.3 million proteins. (Ostlund et al., 2010).

5.8 – Results

5.8.1 – Results - DDB_G0276135

The most intriguing protein of the ones analysed was DDB_G0276135 with gene index number gi|66819173. It was found as a result of three of the BLAST searches; two in the domestic chicken and one in the human liver. It contains a PTHR10783 domain, which according to the Panther database is involved in vacuolar transport (Thomas et al., 2003). Vacuoles in *Dictyostelium discoideum* are known to export calcium into the extracellular medium in response to ATP (Ostlund et al., 2010) and the link between this gene and a system already known to use extracellular ATP signalling was encouraging.

The other domain it contains is PTHR10783. This is known in other organisms to be involved in control of signal transduction and embryonic development. As stated in the introduction, embryonic development and *Dictyostelium* development use many similar pathways (Coates and Harwood, 2001) and control of signal transduction has already been hypothesised in this thesis to be one of the roles that the sought for ecto-ATPase could fulfil.

InParanoid states this gene has similarities to many prespore specific proteins (Ostlund et al., 2010). Genes expressed only in cells destined to become spores must be only expressed during development. This was also encouraging as it has already been suggested within this thesis that the ecto-ATPase has a role in development.

InParanoid gives 14 orthologues of this gene found in 11 different species: *Candida albicans*, *Coccidioides immitis*, *Coprinopsis cinereus*, *Debaryomyces hansenii*, *Fusarium graminearum*, *Magnaporthe grisea*, *Rhizopus oryzae*, *Sclerotinia sclerotiorum*, *Ustilago maydi*, *Yarrowia lipolytica* and *Cyanidioschyzon merolae*. Unfortunately, very few of these species have been the subject of as much detailed genomic work as *Dictyostelium* and as a result the function of most of these orthologues could not be found. In *Candida*, however, one of the orthologues, CAL0003867, is known to have ATP binding activity, and, more importantly, single-stranded DNA-dependent ATPase activity (Inglis et al., 2012).

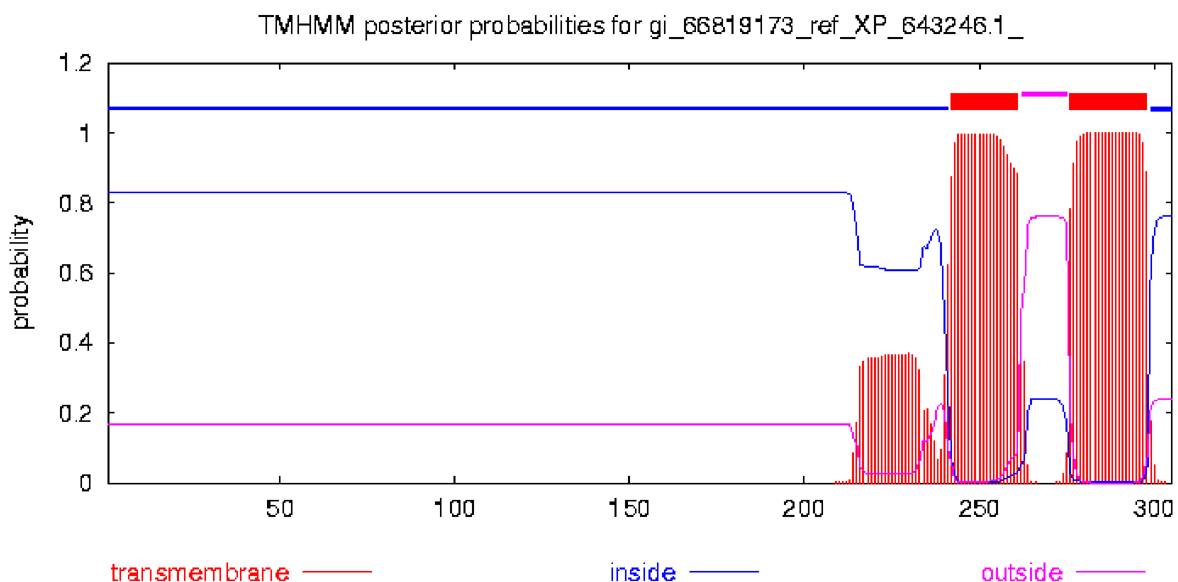


Figure 36 - TMHMM Output For Gene DDB_G0276135 (Analysis, 2007)

There is one key issue, however, that prevents this gene being prioritised for further testing. If one returns to the initial TMHMM readout (Figure 36), one sees that that the extracellular region is very small. In fact it is only 13 amino acids long and this is far too small to contain an ecto-ATPase domain (Analysis, 2007).

The small extra-cellular region of this protein and the fact that its ATPase activity is DNA-dependent implies that, though this protein probably is an ATPase it is not an ecto-ATPase. Its ATPase activity is all internal.

5.8.2 – Results – Other genes

Gene DDB_G0289203 was a product of only one of the BLAST searches, the one carried out using human ectonucleoside triPhosphate diphosphohydrolase 8. It contains the domain of Unknown Function DUF3767 (Gaudet et al., 2011), which means in theory it could be an unknown class of ATPase. It has orthologues in four species; none of which has a known function (Ostlund et al., 2010), though the one in *Tribolium castaneum* is described in Beetle Base as "PREDICTED: similar to lethal (3) 87Df CG7620-PA" (Kim et al., 2010). This is a *Drosophila melanogaster* gene, knock outs of which are known to be lethal (Grumbling and Strelets, 2006). Although potentially it could be an ATPase, it has the same problem as the previous candidate in that it contains only a very small extracellular region, stretching only from amino acids 83-91 (Analysis, 2007).

Gene DDB_G0287477 was a product of five separate BLAST searches. It contains a PTHR31318 domain. Nothing is known about the function of this domain. Five *Dictyostelium discoideum* proteins contain such a domain, though nothing is known about these proteins' functions either (Thomas et al., 2003). Although it is possible that PTHR31318 is an as yet uncharacterised ATPase domain, the Dictybase interface identifies one of the transmembrane regions as containing a signal peptide. A signal peptide is a sequence, which signals to proteases to cut the protein at this location after it has been embedded in the cell's membrane. This structural modification on the outside of the cell allows for the creation of extracellular proteins that might otherwise be unable to pass through the membrane (Dalbey and von Heijne, 2002). In the case of this protein one is left with a single domain anchored in the membrane with the other end allowed to float free. With only one transmembrane region, the chances of it being the sought after ecto-ATPase drops.

Two of the genes (DDB_G0289891 and DDB_G0285083) that were highlighted contained PTHR10919 domains. There are 400 known proteins containing this domain, 231 of them being in *Dictyostelium discoideum*. Panther has no information on the function of this domain and the proteins that contain it seem to carry out a large variety of functions. This makes it hard to draw conclusions as to the primary function of this domain. DDB_G0285083 mutants, however, are known to have aberrant slug formation and cannot form fruiting bodies (Sawai et al., 2007). Neither of these phenotypes would be expected of mutants of the ecto-ATPase from the results already obtained in this thesis.

Gene DDB_G0269622 was a result of only one of the BLAST searches, the one which used the mouse ectonucleoside triPhosphate diphosphohydrolase 7. There is no InParanoid entry for the gene, implying that it doesn't have orthologues in other species. It contains a PTHR24761 domain whose function is unknown. There are 33 known genes containing this domain, three of which are in *Dictyostelium discoideum.* The function of none of these genes is known (Thomas et al., 2003). As a result of this lack of information, there is no evidence that this gene is not an ATPase. Although the only suggestion that it could be one is that it was a result of a BLAST search against a known ecto-ATPase, it could warrant further investigation.

Twelve of the genes contained Epidermal Growth Factor (EGF) Like Domains. Although Epidermal Growth Factors are involved in cell differentiation, like the ATPase being investigated in this study, the family is quite heavily characterised and their function and mechanisms are unrelated to purinergic signalling (Herbst, 2004). They are therefore highly unlikely to have ATPase activity.

Gene DDB_G0283339 was the product of three of the BLAST searches from three very diverse starting sequences from human, *Arabidopsis* and *Xenopus*. It contains a PTHR10529 domain whose function is unknown. According to Panther, most of the genes in other organisms containing this

domain act as molecular signals controlling adhesion during female gamete generation. Furthermore, InParanoid reveals that the orthologues of this gene are also EGF-Like proteins. Both these facts imply that this gene is unlikely to be an ecto-ATPase.

Ten of the genes were identified by InParanoid as being similar to the mouse tenascin genes. Although tenascin is believed to have a role in development, this role is as a membrane anchored attachment point for fibrillins (Szalai et al., 2012). This is not a role likely to be carried out by an ecto-ATPase, so all these proteins were discounted. Six of these proteins contained a PTHR32545 domain. There are seventy proteins known to contain this domain. All of them are found in *Dictyostelium discoideum*. There were four other genes identified by the BLAST searches and TMHMM alignment containing this domain and there was very little other information on any of them. They were discounted from further investigation on the basis that they shared this domain with the tenascin genes which had already been shown to be unlikely to possess ecto-ATPase activity.

Another ten of the genes contained a PTHR24838 domain. In other organisms these domains are found in genes which act as signalling molecules and bind to receptors. It is unlikely that such a domain would be found within an ecto-ATPase so these genes were discarded from the investigation.

Gene DDB_G0283623 contained a PTHR24420 domain and was the result of three BLAST searches, all three from *Arabidopsis* ecto-ATPases. It is the only gene containing such a domain within the *Dictyostelium* genome, but in other organisms it is found within receptors that have protein kinase activity (Thomas et al., 2003). This is again unlikely to be an ATPase.

5.9 – Conclusions

The bioinformatics techniques employed here were unable to identify the *Dictyostelium discoideum* ecto-ATPases. The majority of the genes from the BLAST analysis could be ruled out as being potential ecto-ATPases. DDB_G0289203, however, is similar enough to the human ectonucleoside triPhosphate diphosphohydrolase 8 to be produced in a BLAST search with expect value of 100 and it could not be ruled out. Further investigation into the role it plays could indeed reveal that it is the ecto-ATPase, though a lack of bioinformatic information means that such investigation would have to be carried out using more traditional methods. For example one might knock the gene out and observe whether the phenotype is similar to that of cells developing in the presence of suramin.

If this gene could not be established to be an Ecto-ATPase, then this would imply that *Dictyostelium discoideum* does not express an ecto-ATPase homologous to the ones already known. This is despite the evidence compiled in Chapter 4 and previous work by Parish and Weibel (1980) which suggest that the cells do have ecto-ATPase activity. It therefore seems to be the case that this is carried out by proteins that are non-homologous to the known ecto-ATPases. It should be remembered that the purinergic machinery in *Dictyostelium*, despite carrying out the same functions, only distantly resembles the machinery found in other organisms. The P2X receptors lack a conserved collection of disulphide bridges (Browne et al., 2010) as well as several residues that were previously believed to be essential for ATP binding and which have loss of function knockouts in other organisms (Fountain et al., 2007). It should also be remembered that this project suggests the existence of cell surface ATP receptors, which other bioinformatics projects, including the one that found the internal P2X receptors, failed to find. It, therefore, seems likely that the protein responsible for this extracellular ATP breakdown is too dissimilar to the ecto-ATPases in other organisms to be detected by bioinformatic screenings.

One of the genes which were investigated is believed to be an internal ATPase and, although it is unlikely to be involved in the ATP signalling system investigated in this project, it could still be of interest for further study. *Dictyostelium* is known to have internal ATP signalling systems. Indeed the P2X receptors are all found inside the cell (Sivaramakrishnan and Fountain, 2012) and it could be useful to the field of purinergic signalling if the role of this ATPase could be determined.

Chapter 6 - Discussion

6.1 – An Overview Of The Findings Of This Project

The data presented within this thesis adds to the growing pool of evidence that ATP is used as an extracellular signalling molecule by *Dictyostelium discoideum*. Cells release ATP during both axenic growth and development. Addition of apyrase, an enzyme which degrades ATP and ADP, has no effect upon axenic growth. It does, however, delay the onset of developmental events in both submerged culture and development on agar. In submerged culture apyrase also causes the creation of clumps of cells, though whether these clumps are the cause of the delay, a symptom of it or another unrelated phenotype is unknown. No evidence could be found that these clumps of cells were more adhesive to each other than control cells. It is uncertain whether this phenotype is a result of the loss of one, or both, of these nucleotides or the generation of downstream products of ATP hydrolysis. Experiments have been carried out, however, to suggest that creation of adenosine, a common purinergic molecule and a product of ATP breakdown in mammals, is not responsible for these effects.

Cells treated with apyrase form fewer fruiting bodies than control cells when they develop on agar, but there is no difference in the number of aggregates they form in submerged culture. This implies that the cells treated with apyrase have difficulty transitioning from aggregates to fruiting bodies and this might be linked to the apparently larger number of unaggregated cells in the medium of the wells containing apyrase-treated cells. Cells treated with apyrase produce fruiting bodies with fewer viable cells in the spore heads and it is suggested that inhibiting the cells' own apyrase like enzymes produces fruiting bodies containing more viable cells. Whether these differences in viable cells numbers are a result of changes in cell number in the spore heads, changes in cell viability or whether there is a different cause in each treatment has yet to be determined.

This thesis also suggests that when extracellular ATP is artificially added to *Dictyostelium* cells, extracellular phosphate ions are produced at a certain rate under control conditions. This is hypothesised to be the result of action of the ecto-ATPases which Parish and Weibel characterised in 1980. This phosphate generation appears to be completely inhibited by 250µM suramin and was less effective in the absence of Mg^{2+} ions.

Despite the fact that increased break down of extracellular ATP would induce a variety of developmental effects suramin, a known inhibitor of *Dictyostelium* ecto-ATPases, had no effect on development within submerged culture or on the number of fruiting bodies produced on agar. It did, however, increase the number of viable cells making up these fruiting bodies.

A bioinformatics investigation was launched to try and identify a *Dictyostelium discoideum* ecto-ATPase, but there were no promising candidates. This implies the gene responsible for *Dictyostelium* ecto-ATPase activity is not homologous to known ecto-ATPases.

6.2 –*Dictyostelium discoideum* Responds To Increased Degradation Of Extracellular ATP

This thesis suggests that *Dictyostelium discoideum* releases extracellular ATP into its medium during both the growth and developmental phases of its life cycle. The presence of ecto-ATPases on the cell surface of *Dictyostelium* (Parish and Weibel, 1980) implies the presence of extracellular ADP as well, although this was not tested. The addition of exogenous apyrase influences development, but the apyrase used in these experiments breaks down both ATP and ADP with an equal preference. This leads one to question whether it is breakdown of ATP or of ADP that is responsible for the apyrase treated cells' phenotypes. It is suggested in Chapter 4 that suramin is capable of inhibiting *Dictyostelium* ecto-ATPase activity. If this could be proven to be the case then this might imply that it is the loss of ATP, not ADP, which is responsible. Inhibiting ecto-ATPase activity would decrease the

rate of ATP breakdown but it would also inhibit the rate of ADP generation. If apyrase's breakdown of ADP was responsible for its effect upon development, then one would expect to get a similar effect from treatment with suramin. That the only observed affect that suramin had upon development was the opposite of an effect observed with apyrase, suggests that it is breakdown of ATP that is responsible for the apyrase treated cells' phenotypes.

If it is the loss of ATP or ADP which is responsible for the developmental effects in cells treated with apyrase, then the cells must be capable of detecting the presence, or absence, of ATP. Given the lack of known P2Y receptor analogues in this amoeba, and the fact that all P2X receptors are localised on the inside of the cell (Sivaramakrishnan and Fountain, 2012), the question is raised of how exactly this occurs.

It is conceivable that *Dictyostelium* contains specialised equipment on the cell membrane to transport extracellular nucleotides inside the cell where it can be detected by the P2X receptors. This, however, seems unlikely. Not only does it seem improbably inefficient, but these internal P2X receptors are already known to be involved in release of vesicular calcium into the cytoplasm (Sivaramakrishnan and Fountain, 2012). One assumes that if both systems used the same receptors and the same stimuli, the cells would have the same response to internal ATP as extracellular ATP and this does not appear to be the case.

Another potential, but perhaps controversial, explanation might be that there is another undiscovered class of purinergic receptor located on the *Dictyostelium* cell surface. There is belief amongst some in the purinergic field that more forms of ATP receptors exist. The fact that P2X receptors cannot be found in certain higher organisms, despite ATP signalling having clearly arisen very early in evolution, may suggest that these organisms have evolved alternate methods of detecting ATP making the P2X redundant (Burnstock and Verkhratsky, 2009). There is some evidence from sources other than this project to suggest that *Dictyostelium discoideum* possesses not one, but two, as yet undiscovered receptors sensitive to both ATP and ADP. One resembles the P2X receptor in many ways in terms of pharmacology. When this receptor is inhibited, however, a far smaller and P2Y like response to ATP is still observed (Ludlow et al., 2008). The mechanism of this latter receptor is currently unknown. The fact that in these experiments only simultaneous removal of ATP and adenosine from the development buffer caused a decrease in aggregates could potentially be used as a starting point for further investigation.

A third possible explanation is that *Dictyostelium discoideum* possesses a cell-surface receptor of the classes already known that has until now escaped detection, either another P2X or a P2Y receptor. One assumes that the existence of additional P2X receptors would be unlikely as they would have appeared in the initial bioinformatics screening that revealed the currently known ones. These were, however, only weakly homologous to the other known P2X receptors (Fountain et al., 2007) and this raises the possibility that there are others, which are even more distantly related to the mammalian ones. The idea that *Dictyostelium* possesses a P2X receptor so dissimilar to human P2X receptors as to not be detected initially is plausible, but it seems unlikely that it would possesses a P2X receptor so dissimilar to its own other P2X receptors not to have been revealed by later investigations.

There is, however, perhaps a greater possibility of there being undiscovered P2Y receptors in *Dictyostelium*. It is known to possess a P2Y like response to extracellular ATP (Ludlow et al., 2008) and the experiments presented within this thesis were incapable of differentiating between P2X and P2Y mediated responses. Ludlow's 2008 paper "Purinergic-mediated Ca^{2+} influx in Dictyostelium discoideum" is the only, as of the time of writing, to speculate as to the existence of P2Y receptors in this organism. It therefore remains possible that no bioinformatic screening such as the one in Chapter 5, searching for P2Y receptors as opposed to ecto-ATPases, has yet been completed. Unfortunately, bioinformatics is a field with a high "publication bias;" the phenomena that scientific research which does not produce positive results is less likely to be published (Boulesteix, 2010). An investigation into the presence of P2Y in *Dictyostelium discoideum* might have already been carried out without

producing any candidates (as indeed happened in the bioinformatics investigation within this thesis) and as a result it was never published.

Another possible explanation, again advanced by Ludlow et al., is that the *Dictyostelium* cAMP receptors can potentially be classified as being part of the P2Y family and some could perhaps also be triggered by extracellular ADP. Their results showed that extracellular ATP and ADP activate the same receptor (Ludlow et al., 2008). They suggest that this is a P2Y protein that has already been discovered and classified as a cAMP receptor. They also concede the possibility, however, of yet another ATP receptor's existence in order to explain differences in their results and ones previously observed (Parish and Weibel, 1980) as well as the existence of a P2X-like response to ATP.

A final possibility remains that the changes in developmental timing observed are not a response to the loss of ATP or ADP, but are actually a result of the generation of downstream products of apyrase. In mammalian purinergic signalling systems ATP and ADP are broken down into AMP, which is converted into adenosine by CD73 proteins (Colgan et al., 2013) to activate further purinergic receptors (Burnstock and Verkhratsky, 2009). The generation of adenosine seems unlikely to be responsible for the changes in developmental timing, due to experiment involving treatment of cells simultaneously with apyrase and adenosine deaminase, but these experiments cannot rule out this being an effect of increased generation of AMP. Even if this were the case, however, this would still be indicative of a purinergic signalling system and, in fact, would make these results more important as there is currently no known AMP receptor (Colgan et al., 2013).

Whatever the mechanism, it remains clear that *Dictyostelium discoideum* cells respond to apyrase with either three or four main phenotypical changes, depending on whether the clumping and the delay are the same phenotype or different. What follows is a more detailed consideration of these phenotypes, how the mechanisms behind them could work and how they could be further investigated.

6.3 – Addition Of Apyrase To *Dictyostelium* Causes A Delay In The Initiation Of Streaming And The Formation Of Clumps

The first phenotype to be observed in these experiments was that the addition of apyrase caused a delay in stream formation. The other immediately noticeable effect was that this also caused the formation of clumps of cells. It remains uncertain whether these clumps are the same phenotype or a different one that is triggered by the same conditions.

No evidence could be found that the clumps were a result of cells prematurely adhering to each other. Though absence of evidence is not evidence of absence, this does suggest that the clumps are instead a result of a signalling error. This would in turn imply that transmission of the main signalling molecule, cAMP, is being impaired. Even if the clumps do consist of adhering cells, the fact that they do not form in the adhesion measuring experiment implies that they are dependent on the cAMP waves as clumping does not occur within the constantly mixed tubes.

If the clumps are not a result of adhesion, but cells aggregating together incorrectly, then this could be similar to the multiple conflicting aggregation centres phenotype predicted by Palsson in 2009. This was predicted as a result of imbalances in the ratios between the two forms of cAMP degrading phosphodiesterase. This imbalance causes larger cAMP waves to spread further and undergo constructive interference; resulting in many localised sites of very high cAMP concentration. This could imply that the clumps are a result of interference with cAMP wave propagation and indeed there is already some evidence that extracellular ATP can modulate the size of the cell's response to cAMP (Ludlow et al., 2008). The waves can be visualised under a dark field microscope (Dormann et al., 2000) and it would be interesting to observe the difference in wave formation between control and apyrase-treated cells. Even ignoring the mystery that is clump formation, it would be interesting to

observe the patterns of waves in these circumstances. This would shed light on the progression of development prior to the formation of streams, which is the earliest point in the currently conducted experiments where the developmental effects could be observed.

Dictyostelium discoideum cells are known to condition their media with at least two different proteins, known as cell counting factors, during normal growth. As the cell numbers rise, the density of these proteins also rises. By detecting the levels of these proteins, the cells can ascertain their own density and therefore modulate their responses to stimuli, such as cAMP, accordingly (Van Haastert et al., 1996). If alterations in the cAMP waves are responsible for clump formation, then it is possible that ATP is involved in such a system, either as another cell counting factor or by controlling the rate of creation of one of them. Under this hypothesis the apyrase induced breakdown of ATP would be causing the cells to respond as if the cell density is far lower than it actually is and to produce larger cAMP waves to compensate. The fact, however, that a known inhibitor of *Dictyotelium's* ecto-ATPases, suramin, does not speed up the rate of stream formation seems to contradict this. If ATP was a cell counting factor, then adding suramin would make the cells act as if they were at a higher density. The only way this could be explained is if, as speculated about earlier, the cells cannot respond to higher concentrations of ATP because their control response is already at its maximum. Alternatively if ATP merely controls the rate of release of a cell counting factor, then this could be dependent upon the ATP threshold concentration speculated about at the same time.

It would be useful to attempt to isolate an individual apyrase induced clump from the rest of the cells in order to carry out experiments to ascertain which of these two hypothesises are correct. This might prove difficult to do, however, as control and apyrase-treated cells are both observed to adhere to the base of their wells during development in submerged culture. To inhibit this EDTA would have to be administered, but EDTA has already been shown to have an adverse impact on developing cells (Section 3.7.2). Admittedly, a lower concentration than the one used in the experiments to completely prevent all cell adhesion might have a less harmful effect. Another complication is that, if the clumps are indeed caused by cell-cell adhesion, EDTA would completely destroy them as both the experiments here and by Yang and Brar in 1997 demonstrate that it is very effective at disrupting this. If the clumps are a result of cells adhering to each other and this adhesion is particularly strong, isolation of them might be possible by careful and precise application of pipetting the buffer to dislodge a single clump from the well base and sucking it up. This, however, seems clumsy and unlikely to be successful.

Whatever mechanism is responsible for clump formation, it seems likely that both the observable changes in the cells during submerged culture are a result of alterations to the cAMP waves. It might be tempting to take the evidence of alterations to cAMP waves and the resulting delay in stream formation and conclude that, in the presence of apyrase, the cAMP waves are initiated later or propagate slower, but this is too simplistic a view. Adding apyrase to cells that are already streaming causes the streams to break up. If the delay was truly a result of the stream-initiating cAMP waves starting later, then apyrase would have no effect on already streaming cells. Indeed these cells then go on to form clumps, which could help shed light on the mechanisms of their formation. cAMP is still needed as a signalling molecule during the streaming stage of development. It is transmitted along the length of the streams and released all along them in order to create larger cAMP waves, increase the range of the signal and the number of cells affected. At the same time the differences in local cAMP concentration are being used to guide the already streaming cells towards the centre (Dormann et al., 2001). Perhaps an increase in cAMP release at this stage of development could explain the phenotypes seen here. An increase in the size of the waves released from streams would result in the waves reaching other nearby streams. The cells within them would break off from their own streams in order to follow these new chemotactic impulses. In this scenario clumps would be a result of the formation of multiple aggregation centres, all consisting of only ten or so cells; far too few to form aggregates. Furthermore this would be the direct cause of the delay, as the cells would then need to sort themselves out into a different pattern before aggregation could proceed as normal.

It should be noted that suramin was not observed to have an effect on either the timing of developmental stages or the formation of clumps. This would suggest that either the speed of stream formation is at the highest rate and aggregation quotient is at the lowest value during natural development and that preventing ATP degradation cannot alter this or that the way that ATP degradation affects these traits is not by proportional response, but by being above or below a threshold concentration.

6.4 – Alterations In The Rate Of Breakdown Of Extracellular ATP Causes A Change In The Number Of Viable Cells Making Up *Dictyostelium discoideum* Fruiting Bodies

Addition of apyrase to *Dictyostelium*, which is presumed to cause an in increase in the rate of breakdown of extracellular ATP, caused the fruiting bodies to contain fewer viable cells. Addition of suramin, which is assumed to prevent the cells from breaking down extracellular ATP, caused fruiting bodies to contain more viable cells. From this it seems reasonable to suggest that the number of viable cells within a fruiting body is proportional to the rate of extracellular ATP breakdown.

The question that remains unanswered is whether these differences in the number of viable cells in the fruiting bodies are due to a difference in cell viability or due to a different number of constituent cells. In the discussion of chapter 4, thought is given as to how one might be able to attempt to test these hypothesises.

6.4.1 – The Rate Of ATP Degradation May Affect The Number Of Cells Making Up A Dictyostelium discoideum *Fruiting Body*

If the difference in number of viable cells is due to a difference in the number of cells making up each fruiting body, rather than their viability, then there are three mechanisms known to regulate this. The first of these is the size of the aggregation territory. Smaller aggregation territories mean that fewer cells are able to make up each aggregate and thus smaller aggregates are formed, which consequently develop into smaller fruiting bodies (Jang and Gomer, 2008). Abnormally sized aggregation territories are typically found in cells possessing mutations in secretion of the phosphodiesterases and other genes affecting the cAMP relay. As noted above, there is already evidence to suggest that alterations in cAMP relay might be responsible for the apyrase phenotype. This, however, cannot completely explain the situation. Suramin was not observed to have any phenotype in the submerged culture indicative of modified cAMP waves yet still made a difference in the cell count of the fruiting bodies.

The second mechanism that regulates fruiting body size is cell density or, more accurately, density of the cell counting factors. In high cell densities, streams break up into separate aggregates to ensure that the fruiting bodies do not become too large. In mutants where the counting factors are knocked out, this fails to happen. As a result very large aggregates form and develop into abnormally large fruiting bodies, which are unable to stay upright on their stalks (Brock and Gomer, 1999). This, however, also seems unlikely to be the reason why the fruiting bodies contain different amounts of viable cells when ATP is degraded at different rates. The number of aggregates within each well does not change with the rate of ATP degradation, just the number of viable cells within the eventual fruiting bodies.

The third factor, and probably the most promising in terms of explaining a mechanism for the difference in viable cell number, is that in high cell densities one aggregate can form multiple slugs. Each slug will then develop their own fruiting body. The aggregate must be at a specific size for this to occur. Below this size the aggregate will remain one slug, whereas aggregates larger than this will become multiple (Kopachik, 1982). One assumes this occurs because slugs smaller than this will not contain enough cells to form a fruiting body, but presumably there is some leeway in this.

An aggregate under control conditions might be determined as being too small for this splitting to occur, but with the apyrase induced rate of ATP degradation it could be wrongly determined as being big enough. As a result this fruiting body would split into two slugs, each containing fewer cells and thus produce smaller fruiting bodies. Conversely, by decreasing the rate of ATP degradation (by using suramin), one would expect fewer slugs to be formed from the same number of aggregates and thus each eventual fruiting body to be composed of more cells. This was indeed found to be the case.

Multiple slugs are formed as a result of the creation of multiple mound tips within the same aggregate. The creation of these extra tips can be inhibited by the purinergic molecule adenosine (Jaiswal et al., 2012a). Apyrase generates AMP as its end product and there are mammalian proteins known to further break this down into adenosine (Colgan et al., 2013). If such proteins also existed in *Dictyostelium* to generate adenosine, then suramin would decrease their activity by decreasing the rate of AMP substrate formation. This contradicts the hypothesis. It might be worthwhile to carry out the adenosine deaminase experiments used in submerged culture on cells developing on agar in order to see what affect removal of both ATP and adenosine has on fruiting body size. These results would probably not shed light on the multiple slugs hypothesis, however, because any result is likely to be hidden by the already observed effect that when neither ATP or adenosine are allowed to persist extracellularly, the number of aggregates decreases.

A final factor which should be considered is that apyrase was observed, in submerged culture, to cause fewer cells to commit to aggregation. There being fewer cells capable of development is functionally the same as there being a lower cell density and this could lead to fewer cells making up the same number of aggregates, resulting in smaller fruiting bodies. One would then, however, need to explain why the aggregate number remains the same, despite the functionally smaller cell density. This could possibly be explained by the release of counting factors by these cells prior to their apyrase induced developmental incompetence, as knowledge of the cell density is believed to inform the cells as to the correct number of aggregates that can be supported in an area (Brock and Gomer, 1999).

6.4.2 – The Rate Of ATP Degradation May Affect The Viability Of Cells Making Up A Dictyostelium discoideum *Fruiting Body*

There is another possible explanation for the difference in number of viable cells observed in the fruiting body at different rates of ATP breakdown. This effect may be a result of a change in the viability of the cells within the fruiting body rather the actual number of cells within it. Under this hypothesis the apyrase-treated fruiting bodies consist of mainly unviable cells. This would imply that the transition of the cells from amoeboid free-living cells to spores has been impaired. This hypothesised impairment would not kill the cells as they appear to remain competent enough to carry out the processes needed to be sorted into the fruiting body, but would prevent them from reverting back to normal cells when food became re-available. It is uncertain how such a mechanism might occur, but it has already been observed that there are apyrase treated cells visible within the wells that appear to have lost their developmental capabilities. Perhaps these have undergone the same process as the hypothesised unviable cells within the spores, but at an earlier stage of the developmental process. It would be interesting to test the viability of these unaggregated cells to ascertain whether they are viable cells that didn't develop, unviable cells or dead cells. This could be done by using a pipette to dislodge these cells from the well base, taking care not to dislodge aggregates, plating them onto *Klebsiella* and observing the number of colonies formed. This process is similar to the experiment that ascertained the difference in viable cells in the fruiting bodies in the first place. It should be relatively easy to achieve a volume large enough to calculate their density with the

haemocytometer. The percentage of the cells that are viable could then be compared with that of the (much smaller volume of) undeveloped cells in the control wells.

6.5 – Addition Of Apyrase To *Dictyostelium discoideum* Causes A Decrease In The Number Of Fruiting Bodies Formed.

Addition of apyrase to *Dictyostelium discoideum* cells during development caused a decrease in the number of fruiting bodies formed. One hypothesis suggested to explain this was that apyrase could causes aggregates to split into multiple slugs, despite some of these aggregates being too small for this to happen under control conditions. The result of this would be the creation of slugs that were too small to form fruiting bodies and as a result they would fail to fully develop. Paradoxically the creation of more slugs would actually result in fewer fruiting bodies, which is consistent with the findings of this thesis.

This theory does, however, fail to explain why the ecto-ATPase inhibitor, suramin, does not alter the number of fruiting bodies formed. If in the absence of ATP the cells form more slugs, then one would expect the, assumed, greater ATP concentration in the presence of suramin, to cause fewer aggregates to split. This would in turn cause cells treated with suramin to produce fewer eventual fruiting bodies than control cells and this was not observed to be the case. One cannot suggest an ATP threshold concentration to explain this contradiction, as one has done with other hypothesises within this thesis, because the fact that suramin caused an increase in the number of viable cells within each fruiting body was taken as supporting evidence for this hypothesis.

As discussed earlier, there are many unaggregated cells left over in the apyrase-treated submerged culture wells and one assumes the same of the agar ones. It is entirely possible that the reason for there being fewer fruiting bodies present when cells are treated with apyrase could be a result of fewer cells making up the same number of aggregates. This would raise the likelihood that a given aggregate does not contain enough cells to form a fruiting body. This would in turn result in a lower number of fruiting bodies. This could also explain why suramin has no effect on the fruiting body number, as there were not observed to be a difference in number of unaggregated cells in the wells containing suramin and the control wells. To accept this hypothesis, however, one would then have to explain the increase in viable cells contained within fruiting bodies treated with suramin.

6.6 – What Further Work Could Be Done To Answer The Questions Raised By This Project?

Throughout this thesis, further experiments have been suggested that could help to explain the results obtained and which might prove or disprove hypothesises that had been suggested. For example investigation of the viability of the undeveloped cells within the apyrase treated wells or analysing the mRNA expression of adhesion genes during development. There are, however, other experiments, which are less directly related to specific hypothesises, that could help answer the more general questions raised by this thesis.

With the phenotypes now characterised and an ATP signalling system hypothesised to be responsible for them, work could be done establishing the genetic basis of such a system. The luciferase assay could prove particularly useful here as it able to detect minute differences in ATP concentration. Mass generation of mutants could be carried out and each line grown in a different well of FM minimal media. Once the cells had enough time to increase cell number and condition their media with ATP each well would then be treated with luciferase. Those wells which gave a different luciferase response to the others would contain mutants of genes involved in either the secretion of ATP or in ATPase activity. That aggregates and fruiting bodies formed in all experiments where ATP signalling was impaired suggests that knockouts of these genes would not be lethal. Further tests could then be used to detect where in the genome these mutants differed from the wild type *Dictyostelium* in an attempt to work out which genes are responsible for these roles. Furthermore one could gain

information on the mechanism of the *Dictyostelium* ATP signalling system by observing the phenotypes of these mutants compared to the control strains when developed in submerged culture or on agar.

The ultimate goal of the investigations into ecto-ATPases was to discover the gene that codes for them. If this gene could be identified using other methods, such as the forward genetic luciferase screening suggested above, then genetic recombination techniques could be used to overexpress it. Hypothetically, this strain of *Dictyostelium* would show an apyrase like phenotype. This would show that said effect is not a pharmacological reaction to apyrase, but a true phenotype that cells will display if their intricate ATP signalling machinery is not highly regulated. Discovery of the relevant gene could also lead to greater understanding of how the ATP signalling system works. Is the ATPase constantly expressed or does it only activate when ATP is present in the extracellular medium? This could be determined either by mRNA expression analysis or by attaching a reporter gene upstream of the ATPase in a genetically modified cell line. Sequence analysis of the ATPase, once discovered, would be useful to ascertain why it was not obtained from the bioinformatic assay. If the reason did transpire to be lack of homology to other ecto-ATPases, then a variety of questions about its structure and mechanism would be raised.

One avenue that was considered, but an effective experiment could not be devised, was to investigate whether ATP is involved in pathway preference. When *Dictyostelium discoideum* cells from different culture conditions are mixed together for development the cells tend to end up in the spore heads in an uneven ratio. Although both cultures will form normal, healthy fruiting bodies on their own, factors such as original growth medium, or position in the growth cycle will affect whether a cell is more likely to be a stalk cell or a spore cell with their being a linear hierarchy of cells most likely to be a stalk cell to least likely (MacWilliams et al., 2006). It seems probable that disruptions in ATP signalling could affect a culture's position in the hierarchy. Given that the cells condition their media with ATP prior to development, perhaps one could determine which culture is more likely to be a stalk cell or spore cell when cells grown in FM minimal media and cells grown in apyrase filled FM minimal media were mixed together. A GFP reporter gene could be used to differentiate cells from these two cultures after mixing.

The data accumulated in the course of this thesis shows that the presence and breakdown of extracellular ATP is involved in the regulation of the developmental processes of *Dictyostelium discoideum*. This is despite the absence of either ATP sensing or degrading cell surface proteins homologous to the proteins known to perform these functions in other organisms. The wide variety of functions that purinergic systems are already known to fulfil and the potential implications for human healthcare that have resulted from this imply a need for further research to be carried out in order to explain how exactly *Dictyostelium* extracellular ATP signalling works and whether the system it uses is unique to this amoeba or whether it is shared by higher organisms as well.

References

ABBRACCHIO, M. P., BURNSTOCK, G., BOEYNAEMS, J. M., BARNARD, E. A., BOYER, J. L., KENNEDY, C., KNIGHT, G. E., FUMAGALLI, M., GACHET, C., JACOBSON, K. A. & WEISMAN, G. A. 2006. International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev,* 58**,** 281-341.

ABEDIN, M. & KING, N. 2010. Diverse evolutionary paths to cell adhesion. *Trends Cell Biol,* 20**,** 734-42.

ALBERTS, B. M. 2008. *Molecular Biology of the Cell: Reference Edition*, Taylor & Francis Group.251-253

ALDRICH, S. 2009. *Apyrase from Potato* [Online]. http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/a6410pis.Par.0001.File.tmp/a6410pis.pdf: Sigma Aldrich. [Accessed 10/10/2012].

ANALYSIS, C. F. B. S. 2007. TMHMM Server v. 2.0. http://www.cbs.dtu.dk/services/TMHMM/.

BADER, S., KORTHOLT, A., SNIPPE, H. & VAN HAASTERT, P. J. 2006. DdPDE4, a novel cAMP-specific phosphodiesterase at the surface of dictyostelium cells. *J Biol Chem,* 281**,** 20018-26.

BARICORDI, O. R., FERRARI, D., MELCHIORRI, L., CHIOZZI, P., HANAU, S., CHIARI, E., RUBINI, M. & DI VIRGILIO, F. 1996. An ATP-activated channel is involved in mitogenic stimulation of human T lymphocytes. *Blood,* 87**,** 682-90.

BEIGI, R., KOBATAKE, E., AIZAWA, M. & DUBYAK, G. R. 1999. Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. *Am J Physiol,* 276**,** C267-78.

BORST, P. & ELFERINK, R. O. 2002. Mammalian ABC transporters in health and disease. *Annu Rev Biochem,* 71**,** 537-92.

BOULESTEIX, A. L. 2010. Over-optimism in bioinformatics research. *Bioinformatics,* 26**,** 437-9.

BRACCO, E., PERGOLIZZI, B., PERACINO, B., PONTE, E., BALBO, A., MAI, A., CECCARELLI, A. & BOZZARO, S. 2000. Cell-cell signaling and adhesion in phagocytosis and early development of Dictyostelium. *Int J Dev Biol,* 44**,** 733-42.

BRAR, S., K. & SIU, C. H. 1993. Characterization of the cell adhesion molecule gp24 in Dictyostelium discoideum. Mediation of cell-cell adhesion via a Ca(2+)-dependent mechanism. *J Biol Chem,* 268**,** 24902-9.

BROCK, D. A. & GOMER, R. H. 1999. A cell-counting factor regulating structure size in Dictyostelium. *Genes Dev,* 13**,** 1960–1969.

BROWNE, L. E., JIANG, L. H. & NORTH, R. A. 2010. New structure enlivens interest in P2X receptors. *Trends Pharmacol Sci,* 31**,** 229-37.

BURDINE, V. & CLARKE, M. 1995. Genetic and physiologic modulation of the prestarvation response in Dictyostelium discoideum. *Mol Biol Cell,* 6**,** 311-25.

BURNSTOCK, G. 1972. Purinergic nerves. *Pharmacol Rev,* 24**,** 509-81.

BURNSTOCK, G. & VERKHRATSKY, A. 2009. Evolutionary origins of the purinergic signalling system. *Acta Physiol (Oxf),* 195**,** 415-47.

CHADWICK, B. P. & FRISCHAUF, A. M. 1997. Cloning and mapping of a human and mouse gene with homology to ecto-ATPase genes. *Mamm Genome,* 8**,** 668-72.

CHEN, B. C., LEE, C. M. & LIN, W. W. 1996. Inhibition of ecto-ATPase by PPADS, suramin and reactive blue in endothelial cells, C6 glioma cells and RAW 264.7 macrophages. *Br J Pharmacol,* 119**,** 1628-34.

COATES, J. C. & HARWOOD, A. J. 2001. Cell-cell adhesion and signal transduction during Dictyostelium development. *J Cell Sci,* 114**,** 4349-58.

COLGAN, S. P., FENNIMORE, B. & EHRENTRAUT, S. F. 2013. Adenosine and gastrointestinal inflammation. *J Mol Med (Berl),* 91**,** 157-64.

DALBEY, R. E. & VON HEIJNE, G. 2002. *Protein Targeting, Transport, and Translocation*, Elsevier Science.35-44

DAS, B., MONDRAGON, M. O., SADEGHIAN, M., HATCHER, V. B. & NORIN, A. J. 1994. A novel ligand in lymphocyte-mediated cytotoxicity: expression of the beta subunit of H+ transporting ATP synthase on the surface of tumor cell lines. *J Exp Med,* 180**,** 273-81.

DAS, S., RERICHA, E. C., BAGORDA, A. & PARENT, C. A. 2011. Direct biochemical measurements of signal relay during Dictyostelium development. *J Biol Chem,* 286**,** 38649–38658.

DORMANN, D., KIM, J. Y., DEVREOTES, P. N. & WEIJER, C. J. 2001. cAMP receptor affinity controls wave dynamics, geometry and morphogenesis in Dictyostelium. *J Cell Sci,* 114**,** 2513-23.

DORMANN, D., VASIEV, B. & WEIJER, C. J. 2000. The control of chemotactic cell movement during Dictyostelium morphogenesis. *Philos Trans R Soc Lond B Biol Sci,* 355**,** 983-91.

EICHINGER, L., PACHEBAT, J. A., GLOCKNER, G., RAJANDREAM, M. A., SUCGANG, R., BERRIMAN, M., SONG, J., OLSEN, R., SZAFRANSKI, K., XU, Q., TUNGGAL, B., KUMMERFELD, S., MADERA, M., KONFORTOV, B. A., RIVERO, F., BANKIER, A. T., LEHMANN, R., HAMLIN, N., DAVIES, R., GAUDET, P., FEY, P., PILCHER, K., CHEN, G., SAUNDERS, D., SODERGREN, E., DAVIS, P., KERHORNOU, A., NIE, X., HALL, N., ANJARD, C., HEMPHILL, L., BASON, N., FARBROTHER, P., DESANY, B., JUST, E., MORIO, T., ROST, R., CHURCHER, C., COOPER, J., HAYDOCK, S., VAN DRIESSCHE, N., CRONIN, A., GOODHEAD, I., MUZNY, D., MOURIER, T., PAIN, A., LU, M., HARPER, D., LINDSAY, R., HAUSER, H., JAMES, K., QUILES, M., MADAN BABU, M., SAITO, T., BUCHRIESER, C., WARDROPER, A., FELDER, M., THANGAVELU, M., JOHNSON, D., KNIGHTS, A., LOULSEGED, H., MUNGALL, K., OLIVER, K., PRICE, C., QUAIL, M. A., URUSHIHARA, H., HERNANDEZ, J., RABBINOWITSCH, E., STEFFEN, D., SANDERS, M., MA, J., KOHARA, Y., SHARP, S., SIMMONDS, M., SPIEGLER, S., TIVEY, A., SUGANO, S., WHITE, B., WALKER, D., WOODWARD, J., WINCKLER, T., TANAKA, Y., SHAULSKY, G., SCHLEICHER, M., WEINSTOCK, G., ROSENTHAL, A., COX, E. C., CHISHOLM, R. L., GIBBS, R., LOOMIS, W. F., PLATZER, M., KAY, R. R., WILLIAMS, J., DEAR, P. H., NOEGEL, A. A., BARRELL, B. & KUSPA, A. 2005. The genome of the social amoeba Dictyostelium discoideum. *Nature,* 435**,** 43-57.

FENCKOVA, M., HOBIZALOVA, R., FRIC, Z. F. & DOLEZAL, T. 2011. Functional characterization of ecto-5'-nucleotidases and apyrases in Drosophila melanogaster. *Insect Biochem Mol Biol,* 41**,** 956-67.

FENG, J., CHEN, Y., PU, J., YANG, X., ZHANG, C., ZHU, S., ZHAO, Y., YUAN, Y., YUAN, H. & LIAO, F. 2011. An improved malachite green assay of phosphate: mechanism and application. *Anal Biochem,* 409**,** 144-9.

FLEGEL, K. A., PINEDA, J. M., SMITH, T. S., LASZCZYK, A. M., PRICE, J. M., KARASIEWICZ, K. M. & DAMER, C. K. 2011. Copine A is expressed in prestalk cells and regulates slug phototaxis and thermotaxis in developing Dictyostelium. *Dev Growth Differ,* 53**,** 948-59.

FOUNTAIN, S. J., PARKINSON, K., YOUNG, M. T., CAO, L., THOMPSON, C. R. & NORTH, R. A. 2007. An intracellular P2X receptor required for osmoregulation in Dictyostelium discoideum. *Nature,* 448**,** 200-3.

FRANKE, J. & KESSIN, R. 1977. A defined minimal medium for axenic strains of Dictyostelium discoideum. *Proc Natl Acad Sci U S A,* 74**,** 2157-61.

GAUDET, P., FEY, P., BASU, S., BUSHMANOVA, Y. A., DODSON, R., SHEPPARD, K. A., JUST, E. M., KIBBE, W. A. & CHISHOLM, R. L. 2011. dictyBase update 2011: web 2.0 functionality and the initial steps towards a genome portal for the Amoebozoa. *Nucleic Acids Res,* 39**,** D620-4.

GILBERT , O. M., FOSTER, K. R., MEHDIABADI, N. J., STRASSMANN, J. E. & QUELLER, D. C. 2007. High relatedness maintains multicellular cooperation in a social amoeba by controlling cheater mutants. *Proc Natl Acad Sci U S A,* 104**,** 8913-7.

GRINTHAL, A. & GUIDOTTI, G. 2006. CD39, NTPDase 1, is attached to the plasma membrane by two transmembrane domains. Why? *Purinergic Signal,* 2**,** 391-8.

GRUMBLING, G. & STRELETS, V. 2006. FlyBase: anatomical data, images and queries. *Nucleic Acids Res,* 34**,** D484-8.

HAM, J. & EVANS, B. A. 2012. An emerging role for adenosine and its receptors in bone homeostasis. *Front Endocrinol (Lausanne),* 3**,** 113.

HERBST, R. S. 2004. Review of epidermal growth factor receptor biology. *Int J Radiat Oncol Biol Phys,* 59**,** 21-6.
INGLIS, D. O., ARNAUD, M. B., BINKLEY, J., SHAH, P., SKRZYPEK, M. S., WYMORE, F., BINKLEY, G., MIYASATO, S. R., SIMISON, M. & SHERLOCK, G. 2012. The Candida genome database incorporates multiple Candida species: multispecies search and analysis tools with curated gene and protein information for Candida albicans and Candida glabrata. *Nucleic Acids Res,* 40**,** D667-74.
JAIN, R., YUEN, I. S., TAPHOUSE, C. R. & GOMER, R. H. 1992. A density-sensing factor controls development in Dictyostelium. *Genes Dev,* 6**,** 390-400.
JAISWAL, P., SINGH, S. P., AIYAR, P., AKKALI, R. & BASKAR, R. 2012a. Regulation of multiple tip formation by caffeine in cellular slime molds. *BMC Dev Biol,* 12**,** 26.
JAISWAL, P., SOLDATI, T., THEWES, S. & BASKAR, R. 2012b. Regulation of aggregate size and pattern by adenosine and caffeine in cellular slime molds. *BMC Dev Biol,* 12.
JANG, W. & GOMER, R. H. 2008. Combining experiments and modelling to understand size regulation in Dictyostelium discoideum. *J R Soc Interface,* 5 Suppl 1**,** S49-58.
JIANG, R., TALY, A., LEMOINE, D., MARTZ, A., CUNRATH, O. & GRUTTER, T. 2012. Tightening of the ATP-binding sites induces the opening of P2X receptor channels. *EMBO J,* 31**,** 2134-2143.
KAHSAY, R. Y., GAO, G. & LIAO, L. 2005. An improved hidden Markov model for transmembrane protein detection and topology prediction and its applications to complete genomes. *Bioinformatics,* 21**,** 1853-8.
KIM, H. S., MURPHY, T., XIA, J., CARAGEA, D., PARK, Y., BEEMAN, R. W., LORENZEN, M. D., BUTCHER, S., MANAK, J. R. & BROWN, S. J. 2010. BeetleBase in 2010: revisions to provide comprehensive genomic information for Tribolium castaneum. *Nucleic Acids Res,* 38**,** D437-42.
KNOWLES, A. F. 2011. The GDA1_CD39 superfamily: NTPDases with diverse functions. *Purinergic Signal,* 7**,** 21-45.
KOPACHIK, W. 1982. Size regulation in Dictyostelium. *J Embryol Exp Morphol,* 68**,** 23-35.
KORTHOLT, A., KATARIA, R., KEIZER-GUNNINK, I., VAN EGMOND, W. N., KHANNA, A. & VAN HAASTERT, P. J. 2011. Dictyostelium chemotaxis: essential Ras activation and accessory signalling pathways for amplification. *EMBO Rep,* 12**,** 1273-9.
KUKULSKI, F., LEVESQUE, S. A., LAVOIE, E. G., LECKA, J., BIGONNESSE, F., KNOWLES, A. F., ROBSON, S. C., KIRLEY, T. L. & SEVIGNY, J. 2005. Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. *Purinergic Signal,* 1**,** 193-204.
KUZDZAL-FICK, J. J., QUELLER, D. C. & STRASSMANN, J. E. 2010. An invitation to die: initiators of sociality in a social amoeba become selfish spores. *Biol Lett,* 6**,** 800-802.
LARKIN, M. A., BLACKSHIELDS, G., BROWN, N. P., CHENNA, R., MCGETTIGAN, P. A., MCWILLIAM, H., VALENTIN, F., WALLACE, I. M., WILM, A., LOPEZ, R., THOMPSON, J. D., GIBSON, T. J. & HIGGINS, D. G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics,* 23**,** 2947-8.
LOHMAN, A. W., BILLAUD, M. & ISAKSON, B. E. 2012. Mechanisms of ATP release and signalling in the blood vessel wall. *Cardiovasc Res,* 95**,** 269-80.
LUDLOW, M. J., DURAI, L. & ENNION, S. J. 2009. Functional characterization of intracellular Dictyostelium discoideum P2X receptors. *J Biol Chem,* 284**,** 35227-39.
LUDLOW, M. J., TRAYNOR, D., FISHER, P. R. & ENNION, S. J. 2008. Purinergic-mediated Ca2+ influx in Dictyostelium discoideum. *Cell Calcium,* 44**,** 567-79.
MACWILLIAMS, H., DOQUANG, K., PEDROLA, R., DOLLMAN, G., GRASSI, D., PEIS, T., TSANG, A. & CECCARELLI, A. 2006. A retinoblastoma ortholog controls stalk/spore preference in Dictyostelium. *Development,* 7**,** 1287-97.
MAMMANO, F. 2012. ATP-dependent intercellular Ca(2+) signaling in the developing cochlea: facts, fantasies and perspectives. *Semin Cell Dev Biol,* 24**,** 31-9.
MANAHAN, C. L., IGLESIAS, P. A., LONG, Y. & DEVREOTES, P. N. 2004. Chemoattractant signaling in dictyostelium discoideum. *Annu Rev Cell Dev Biol,* 20**,** 223-53.

MATHIEU, P. 2012. Pharmacology of ectonucleotidases: Relevance for the treatment of cardiovascular disorders. *Eur J Pharmacol,* 696**,** 1-4.

MATO, J. M. & KONIJN, T. M. 1975. Enhanced cell aggregation in Dictyostelium discoideum by ATP activation of Cyclic AMP receptors. *Dev Biol,* 47**,** 233-5.

MIN, J., SRIDEVI, P., ALEXANDER, S. & ALEXANDER, H. 2006. Sensitive cell viability assay for use in drug screens and for studying the mechanism of action of drugs in Dictyostelium discoideum. *Biotechniques,* 41**,** 591-5.

MONTAGNES, D., ROBERTS, E., LUKES, J. & LOWE, C. 2012. The rise of model protozoa. *Trends Microbiol,* 20**,** 184-91.

MUTAFOVA-YAMBOLIEVA, V. N. 2012. Neuronal and extraneuronal release of ATP and NAD(+) in smooth muscle. *IUBMB Life,* 64**,** scope.

NCBI.NLM.NIH.GOV. *BLAST Frequently Asked questions.* [Online]. http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=FAQ. [Accessed 03/10/2012].

NICHOLS, W. W., CURTIS, G. D. & JOHNSTON, H. H. 1981. The identity and properties of firefly luciferase inhibitors in urine. *Anal Biochem,* 114**,** 433-40.

NORATEL, E. F., PETTY, C. L., KELSEY, J. S., COST, H. N., BASAPPA, N. & BLUMBERG, D. D. 2012. The adhesion modulation protein, AmpA localizes to an endocytic compartment and influences substrate adhesion, actin polymerization and endocytosis in vegetative Dictyostelium cells. *BMC Cell Biol,* 13**,** 29.

OSTLUND, G., SCHMITT, T., FORSLUND, K., KOSTLER, T., MESSINA, D. N., ROOPRA, S., FRINGS, O. & SONNHAMMER, E. L. 2010. InParanoid 7: new algorithms and tools for eukaryotic orthology analysis. *Nucleic Acids Res,* 38**,** D196-203.

PALSSON, E. 2009. A cAMP signaling model explains the benefit of maintaining two forms of phosphodiesterase in Dictyostelium. *Biophys J,* 97**,** 2388-98.

PARISH, R. W. 1977. Inhibition of Dictyostelium discoideum beta-glucosidase by purines. *J Bacteriol,* 129**,** 1642-4.

PARISH, R. W. & WEIBEL, M. 1980. Extracellular ATP, ecto-ATPase and calcium influx in Dictyostelium discoideum cells. *FEBS Lett,* 118**,** 263-6.

PELLEGATTI, P., RAFFAGHELLO, L., BIANCHI, G., PICCARDI, F., PISTOIA, V. & DI VIRGILIO, F. 2008. Increased Level of Extracellular ATP at Tumor Sites: <italic>In Vivo</italic> Imaging with Plasma Membrane Luciferase. *PLoS ONE,* 3**,** e2599.

RALEVIC, V. & BURNSTOCK, G. 1998. Receptors for purines and pyrimidines. *Pharmacol Rev,* 50**,** 413-92.

RAY, S., CHEN, Y., AYOUNG, J., HANNA, R. & BRAZILL, D. 2011. Phospholipase D controls Dictyostelium development by regulating G protein signaling. *Cell Signal,* 23**,** 335-43.

RIEWE, D., GROSMAN, L., FERNIE, A. R., WUCKE, C. & GEIGENBERGER, P. 2008. The potato-specific apyrase is apoplastically localized and has influence on gene expression, growth, and development. *Plant Physiol,* 147**,** 1092-109.

ROBERTS, J. A., VIAL, C., DIGBY, H. R., AGBOH, K. C., WEN, H., ATTERBURY-THOMAS, A. & EVANS, R. J. 2006. Molecular properties of P2X receptors. *Pflugers Arch,* 452**,** 486-500.

ROBSON, S. C., SEVIGNY, J. & ZIMMERMANN, H. 2006. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. *Purinergic Signal,* 2**,** 409-30.

SABOURY, A. A., DIVSALAR, A., JAFARI, G. A., MOOSAVI-MOVAHEDI, A. A., HOUSAINDOKHT, M. R. & HAKIMELAHI, G. H. 2002. A product inhibition study on adenosine deaminase by spectroscopy and calorimetry. *J Biochem Mol Biol,* 35**,** 302-5.

SAWAI, S., GUAN, X. J., KUSPA, A. & COX, E. C. 2007. High-throughput analysis of spatio-temporal dynamics in Dictyostelium. *Genome Biol,* 8**,** R144.

SAXER, G., BROCK, D. A., QUELLER, D. C. & STRASSMANN, J. E. 2010. Cheating does not explain selective differences at high and low relatedness in a social amoeba. *BMC Evol Biol,* 10**,** 76.

SCHAAP, P. 2011. Evolution of developmental cyclic adenosine monophosphate signaling in the Dictyostelia from an amoebozoan stress response. *Dev Growth Differ,* 53**,** 452-462.
SESAKI, H. & SIU, C. H. 1996. Novel redistribution of the Ca(2+)-dependent cell adhesion molecule DdCAD-1 during development of Dictyostelium discoideum. *Dev Biol,* 177**,** 504-16.
SHERWOOD, L. 2012. *Human Physiology: From Cells to Systems,* Belmont, CA, USA, Cengage Learning.28-29
SIEGERT, F. & WEIJER, C. J. 1995. Spiral and concentric waves organize multicellular Dictyostelium mounds. *Curr Biol,* 5**,** 937-43.
SISSONS, J., ALSAM, S., JAYASEKERA, S. & KHAN, N. A. 2004. Ecto-ATPases of clinical and non-clinical isolates of Acanthamoeba. *Microb Pathog,* 37**,** 231-239.
SIU, C.-H., WONG, L. M., CHOI, A. & CHO, A. 1988. Mechanisms of cell-cell recognition and cell cohesion in *Dictyostelium discoideum* cells. *In:* G.P., C., C.C., A. & C.J., C. (eds.) *Eukaryote cell recognition: concepts and model systems.* Cambridge: Cambridge University Press.
SIVARAMAKRISHNAN, V. & FOUNTAIN, S. J. 2012. A Mechanism of Intracellular P2X Receptor Activation. *J Biol Chem,* 287**,** 28315-26.
SRISKANTHADEVAN, S., ZHU, Y., MANOHARAN, K., YANG, C. & SIU, C. H. 2011. The cell adhesion molecule DdCAD-1 regulates morphogenesis through differential spatiotemporal expression in Dictyostelium discoideum. *Development,* 138**,** 2487-97.
STERNFELD, J. & BONNER, J. T. 1977. Cell differentiation in Dictyostelium under submerged conditions. *Proc Natl Acad Sci U S A,* 74**,** 268-71.
SURPRENANT, A. & NORTH, R. A. 2009. Signaling at Purinergic P2X Receptors. *Annual Review of Physiology.* Palo Alto: Annual Reviews.
SZALAI, E., FELSZEGHY, S., HEGYI, Z., MODIS, L., JR., BERTA, A. & KAARNIRANTA, K. 2012. Fibrillin-2, tenascin-C, matrilin-2, and matrilin-4 are strongly expressed in the epithelium of human granular and lattice type I corneal dystrophies. *Mol Vis,* 18**,** 1927-36.
TATUR, S., GROULX, N., ORLOV, S. N. & GRYGORCZYK, R. 2007. Ca2+-dependent ATP release from A549 cells involves synergistic autocrine stimulation by coreleased uridine nucleotides. *J Physiol,* 584**,** 419-35.
THOMAS, P. D., KEJARIWAL, A., CAMPBELL, M. J., MI, H., DIEMER, K., GUO, N., LADUNGA, I., ULITSKY-LAZAREVA, B., MURUGANUJAN, A., RABKIN, S., VANDERGRIFF, J. A. & DOREMIEUX, O. 2003. PANTHER: a browsable database of gene products organized by biological function, using curated protein family and subfamily classification. *Nucleic Acids Res,* 31**,** 334-341.
THOMPSON, J. D., HIGGINS, D. G. & GIBSON, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res,* 22**,** 4673-80.
TURMAN, M. A. & MATHEWS, A. 1996. A simple luciferase assay to measure atp levels in small numbers of cells using a fluorescent plate reader. *In Vitro Cell Dev Biol Anim,* 32**,** 1-4.
TYLER, M. S. 2003. *Developmental Biology: A Guide for Experimental Studies* Sinauer Associates, Inc., 23 Plumtree Road, Sunderland, Massachusetts Sinauer Assoc.48-52
VAN HAASTERT, P. J., BISHOP, J. D. & GOMER, R. H. 1996. The cell density factor CMF regulates the chemoattractant receptor cAR1 in Dictyostelium. *J Cell Biol,* 134**,** 1543-9.
VELICER, G. J. 2005. Evolution of cooperation: does selfishness restraint lie within? *Curr Biol,* 15**,** R173-5.
VELTMAN, D. M. & VAN HAASTERT, P. J. 2008. The role of cGMP and the rear of the cell in Dictyostelium chemotaxis and cell streaming. *J Cell Sci,* 121**,** 120-127.
VON KÜGELGEN, I. & WETTER, A. 2000. Molecular pharmacology of P2Y-receptors. *Naunyn-Schmiedeberg's Archives of Pharmacology,* 362**,** 310-323.
WEIJER, C. J. 2009. Collective cell migration in development. *J Cell Sci,* 122**,** 3215-23.
YAMADA, Y., MINAMISAWA, H., FUKUZAWA, M., KAWATA, T. & OOHATA, A. A. 2010. Prespore cell inducing factor, psi factor, controls both prestalk and prespore gene expression in Dictyostelium development. *Dev Growth Differ,* 52**,** 377-83.

YANG, C., BRAR, S., K., DESBARATS, L. & SIU, C. H. 1997. Synthesis of the Ca(2+)-dependent cell adhesion molecule DdCAD-1 is regulated by multiple factors during Dictyostelium development. *Differentiation,* 61**,** 275-84.

ZHANG, H., GOMEZ-GARCIA, M. R., BROWN, M. R. & KORNBERG, A. 2005. Inorganic polyphosphate in Dictyostelium discoideum: influence on development, sporulation, and predation. *Proc Natl Acad Sci U S A,* 102**,** 2731-5.

ZIMMERMANN, H., BRAUN, N., KEGEL, B. & HEINE, P. 1998. New insights into molecular structure and function of ecto-nucleotidases in the nervous system. *Neurochemistry International,* 32**,** 421-425.

Appendix - Dictyostelium Genes Identified By The BLAST Analysis That Had Been Determined To Contain Two Transmembrane Domains And The Correct Topological Alignment

Dictybase Gene name	Pubmed Gene ID	Number of Blast Searches that produced this protein	Sequence(s) Input to Blast	Domains	Inparanoid Data
DDB_G0270488	gi|66825099	11	gi|14547943, gi|148232431, gi|47523398, gi|62859996, gi|6753346, gi|74039689, gi|12018242, gi|16518970, gi|45383632, gi|45827718, gi|57525937	PTHR24838, Concanavalin A-like lectins/glucanases, laminin and EGFs	N/a
DDB_G0288955	gi|66805509	6	gi|12018242, gi|358440048, gi|45580700, gi|47523398, gi|6753346, gi|296472657	PTHR24838, Serine protease inhibitors and EGFs, Calcium ion binding	Fibrillins and related proteins containing Ca2+-binding EGF-like domains
DDB_G0293722	gi|66800229	6	gi|14547943, gi|74231263, gi|9966821, gi|207080274, gi|16518970, gi|45383632	PTHR32545, Signal peptide	EGF-like domain-containing protein, germination protein
DDB_G0280125	gi|66814156	5	gi|27806971, gi|296472657, gi|50539906, gi|18093090, gi|358440048	Leishmanolysin-Like Peptidase	Gp63 homolog
DDB_G0283675	gi|66810496	5	gi|16758256, gi|207080274, gi|358440048, gi|82617566, gi|9966821	Signal peptide	N/a
DDB_G0287477	gi|66806981	5	gi|12018242, gi|30017439, gi|74039691, gi|75677557, gi|57525937	PTHR31318	N/a
DDB_G0269556	gi|66825419	4	gi|30017439, gi|59003436, gi|75677557, gi|110431368	Just a transmembrane, no functional groups	N/a

DDB_G0280501	gi|66814062	4	gi|207080274, gi|82617566, gi|9966821	Signal peptide	N/a
DDB_G0281231	gi|66813440	4	gi|16518970, gi|45383632, gi|50539906, gi|14547943	PTHR24838	EGF-like domain-containing protein
DDB_G0287879	gi|66806611	4	gi|12018242, gi|45580700, gi|47523398, gi|6753346	Ring U box	N/a
DDB_G0270708	gi|66826263	3	gi|339283646, gi|170791248, gi|6753346	PTHR32545	Similar to Mus musculus (Mouse). tenascin C.
DDB_G0272714	gi|66823023	3	gi|16518970, gi|45383632, gi|14547943	Just a transmembrane, no functional groups	N/a
DDB_G0275747	gi|66819765	3	gi|207080274, gi|45580700, gi|9966821	Signal Peptide	FG-GAP repeat-containing protein
DDB_G0275813	gi|66819491	3	gi|148232431, gi|74039689, gi|74231265	Mitochondrial mRNA splicing protein, Magnesium Transporter	Similar to putative mitochondrial rna splicing protein
DDB_G0276135	gi|66819173	3	gi|16518970, gi|45383632, gi|14547943	Domain of unknown function. Known to be part of a g-protein linked signal pathway involved in embryonic development and vacuolar transport	Known to be a prespore specific protein in Dictyostelium
DDB_G0277555	gi|66817578	3	gi|16518970, gi|45383632, gi|14547943	PTHR32545	Similar to Mus musculus (Mouse). tenascin C.
DDB_G0277557	gi|66817580	3	gi|296481999, gi|110431368, gi|59003436	PTHR32545	Similar to Mus musculus (Mouse). tenascin C.
DDB_G0280511	gi|66814074	3	Conserved Region 3, gi|57525937, gi|74231265	PTHR32545, EGF like domains	Similar to Mus musculus (Mouse). tenascin C, EGF-like domain-containing protein
DDB_G0283339	gi|66810896	3	gi|339283652, gi|134085336, gi|358440048	Low-Density Lipoprotein Receptor-Related	germination protein, EGF-like domain-containing protein
DDB_G0283623	gi|66810397	3	gi|339283646, gi|339283648, gi|339283650	PTHR24420	Similar to Mus musculus (Mouse). tenascin C

DDB_G0286877	gi|66807557	3	gi|148232431, gi|161484610, gi|74039689	Domains uncharacterised	N/a
DDB_G0289113	gi|66805353	3	gi|207080274, gi|82617566, gi|9966821	Immunoglobulin fold, PTHR32545, EGF Laminin, E-set domains	EGF-like domain-containing protein
DDB_G0289891	gi|66804353	3	gi|194369341, gi|194369341, gi|57525937	PTHR10919	Rhizopus oryzae predicted protein
DDB_G0270666	gi|66825971	2	gi|30017439, gi|82617566	Serine-Threonine Protein Kinase	GDT family protein kinase
DDB_G0272396	gi|66823673	2	gi|45120586, gi|45120586	Signal Peptide	N/a
DDB_G0276243	gi|66819301	2	gi|71061460, gi|134133300	PTHR21573, Quinoprotein alcohol dehydrogenase-like	Conserved in several species, mainly arabidopsis. Function undetermined
DDB_G0287035	gi|66807353	2	gi|59003436, gi|110431368	CD36, Scavenger Receptor Class B Type-1, Lysosomal Integral Membrane Protein II	lysosomal integral membrane protein II
DDB_G0287833	gi|66806725	2	gi|296481999, gi|30017439	CD166 Antigen	N/a
DDB_G0288713	gi|66805739	2	gi|12018242, gi|161484610	Just a transmembrane, no functional groups	N/a
DDB_G0293066	gi|66800753	2	gi|339283650, gi|223649432	Signal peptide, Conserved in Dicty purpureum,	N/a
DDB_G0290635	gi|66803747	1	Conserved Region 3	PSEUDOGENE	PSEUDOGENE
DDB_G0267894	gi|66828073	1	gi|300795252	Just a transmembrane, no functional groups	N/a
DDB_G0267896	gi|66828075	1	gi|339283652	Golgin subfamily member, function unknown	N/a
DDB_G0268314	gi|66827749	1	gi|74039691	PTHR24032, EGF Like domains	Similar to Mus musculus (Mouse). tenascin C.
DDB_G0269596	gi|66825489	1	gi|18093090	Just a transmembrane, no functional groups	N/a
DDB_G0269622	gi|66825537	1	gi|82617566	PTHR24761	N/a
DDB_G0270438	gi|66826899	1	gi|62859996	NADH-ubiquinone	putative transmembrane protein

				oxidoreductase, 21kDa subunit	
DDB_G0270608	gi|66825651	1	gi|45580700	DELETED FROM DICTYBASE	DELETED FROM DICTYBASE
DDB_G0271290	gi|66824635	1	gi|82617566	Signal Peptide	N/a
DDB_G0271638	gi|66824459	1	gi|45384424	PTHR32545, EGF like domains	N/a
DDB_G0278651	gi|111226726	1	gi|339283652	ALPHA-MANNOSIDASE	N/a
DDB_G0278677	gi|66816645	1	gi|62859996	Just a transmembrane, no functional groups	N/a
DDB_G0278879	gi|111226669	1	gi|62859996	Serine-Threonine Protein Kinase, TGF-BETA-Activated Kinase 1	GDT family protein kinase
DDB_G0279815	gi|66814638	1	gi|74039691	Signal Peptide	N/a
DDB_G0281067	gi|66813234	1	Conserved Region 3	Signal Peptide	N/a
DDB_G0281447	gi|66813006	1	Conserved Region 6	Signal Peptide	Orthologues in several species
DDB_G0282201	gi|66812208	1	gi|47523398	Signal Peptide	N/a
DDB_G0282947	gi|66811130	1	gi|45120584	ADAMTS-Like domain	N/a
DDB_G0283743	gi|66810474	1	gi|74039691	Signal Peptide	N/a
DDB_G0283869	gi|66810273	1	gi|112821698	PTHR32545, EGF like domains	Similar to Mus musculus (Mouse). tenascin C., EGF Like domain
DDB_G0285229	gi|66809235	1	Conserved Region 3	Just Transmembrane Regions, no recognised Domain	N/a
DDB_G0285585	gi|66808837	1	gi|166197702	Sugar-1-Phosphate Guanyl Transferase, Eukariotic Translation Initiation Factor 2B, Epsilon Subunit, EGF Like Domains	Similar to Mus musculus (Mouse). tenascin X.
DDB_G0285987	gi|66808467	1	gi|339283646	Just a transmembrane, no functional groups	N/a
DDB_G0286455	gi|66808033	1	gi|12018242	MRNA export factor MEX67, nuclear RNA	Conserved in several species. Function undetermined

				export factor	
DDB_G0286541	gi|66807911	1	Conserved Region 2	TIM23	mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family
DDB_G0287193	gi|66807271	1	gi|339283648	Signal Peptide	N/a
DDB_G0287329	gi|66807037	1	Conserved Region 1	Signal Peptide	N/a
DDB_G0287385	gi|66807117	1	Conserved Region 3	Just Transmembrane Regions, no recognised Domain	N/a
DDB_G0288843	gi|66805663	1	gi|170791250	PSEUDOGENE	PSEUDOGENE
DDB_G0288849	gi|66805669	1	gi|75677557	PTHR1261, Endoplasmic reticulum oxidoreductin 1	Conserved in several species. Function undetermined
DDB_G0288863	gi|66805589	1	Conserved Region 1	1-Acylglycerol-3-Phosphate Acyltransferase-Related	N/a
DDB_G0289203	gi|66805205	1	gi|110431368	Domain of unknown function	Conserved in Strongylocentrotus purpuratus
DDB_G0290841	gi|66803352	1	gi|82617566	PTHR32545	Similar to Mus musculus (Mouse). tenascin X, EGF Like Domains
DDB_G0291045	gi|66803238	1	Plant Conserved Regions	PTHR32545	N/a
DDB_G0292032	gi|66801709	1	Conserved Region 3	Carboxypeptidase regulatory domain-like, Starch-binding domain-like	Metalloproteinase-related collagenase pM5, carbohydrate binding
DDB_G0292098	gi|66802009	1	gi|12018242	PTHR32545	Similar to Mus musculus (Mouse). tenascin C.
DDB_G0292944	gi|66800987	1	gi|50539906	DELETED FROM DICTYBASE	DELETED FROM DICTYBASE
DDB_G0295675	gi|166240261	1	gi|339283648	Interleukin-1 Receptor-Associated Kinase	N/a