# Role of $Ca^{2+}$ Release and $Ca^{2+}$ Entry Mechanisms During Pathology and The Initiation of Acute Pancreatitis

Thesis submitted in accordance with the requirements of Cardiff University for the degree of Doctor of Philosophy


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# Abstract

***Introduction –*** Acute pancreatitis is an inflammatory and deadly disease affecting the pancreas which is strongly associated with pathological calcium signals within pancreatic exocrine cells. These pathological global transient signals involve several mechanisms of calcium signalling including calcium release via inositol triphosphate and ryanodine receptors as well as calcium entry via store operated calcium channels. Pathological calcium signals induce necrotic effects triggered by pathological stimuli such as the bile acid, TLC-S and asparaginase, a drug commonly used to treat acute lymphoblastic leukaemia. Therefore, our goal is to investigate pathological calcium signals to prevent the development of acute pancreatitis.

Previous studies have demonstrated the beneficial uses of inhibitors and compounds such as CM-4620, currently in human trials, GSK-7975A and NED-19, which are useful pharmacological tools to investigate calcium entry and NAADP-mediated calcium signalling respectively. There has been recent interest in calcium signalling regulator proteins and peptides, such as BH4-Bcl-2, and energy supplements, such as galactose which aids cell survival by reducing ATP loss, as potential therapeutic strategies. Moreover, the BH4 domain of the Bcl-2 protein has demonstrated regulation of ryanodine and inositol triphosphate receptors. This thesis focuses on the mechanisms of calcium release and entry effects of pathological stimuli within pancreatic acinar and stellate cells.

***Results –*** Using confocal microscopy techniques we have demonstrated the detrimental effects pathological agents have upon mitochondria within pancreatic acinar cells and the potential protective effects of BH4-Bcl-2 treatments upon mitochondria and cell death. The pathological increase in mitochondrial calcium reduces ATP production, increasing the likelihood of cell death and induction of acute pancreatitis. BH4-Bcl-2 demonstrated protective effects upon pathological calcium signalling and necrosis during both TLC-S and asparaginase stimulation. Furthermore, the protective effects of BH4-Bcl-2 were also granted when reducing the concentration of applied BH4-Bcl-2 peptide given in combination with galactose.

Additionally, this study demonstrated reduced calcium entry due to NED-19 treatment suggesting possible linkage between calcium entry and NAADP-mediated signalling. Therefore, suggesting its potential use as a therapeutic against pathological calcium signalling reliant upon calcium entry during sustained global responses.

***Conclusion –*** Overall, this thesis provides new and additional evidence for the use of the BH4-Bcl-2 peptide to counteract pancreatic pathology at the cellular and mitochondrial level. Furthermore, it presents new arguments to use combinatory treatments in future studies. Additionally, this thesis reports inhibition of calcium entry by NED-19 that requires further investigation into NAADP-induced calcium signalling.

*This thesis is dedicated to my parents for their support throughout my endeavours*

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# List of Abbreviations

| | |
|---|---|
| $[Ca^{2+}]$ | Calcium Concentration |
| $[Ca^{2+}]_{ER}$ | Endoplasmic Reticulum Concentration |
| $[Ca^{2+}]_i$ | Cytosolic Calcium Concentration |
| $[Ca^{2+}]_m$ | Mitochondrial Calcium Concentration |
| ACh | Acetylcholine |
| ADH | Alcohol Dehydrogenase |
| ADP | Adenosine Diphosphate |
| AP | Acute Pancreatitis |
| ASNase | L-Asparaginase |
| ATP | Adenosine Triphosphate |
| $Ca^{2+}$ | Calcium Ions |
| cADPR | Cyclic Adenosine Diphosphate Ribose |
| CaM | Calmodulin |
| cAMP | Cyclic Adenosine Monophosphate |
| CaSR | Calcium-Sensing Receptor |
| CCK | Cholecystokinin |
| CFTR | Cystic Fibrosis Transmembrane Conductance Regulator |
| CICR | Calcium Induced Calcium Release |
| CP | Chronic Pancreatitis |
| CPA | Cyclopiazonic Acid |
| CRAC | $Ca^{2+}$ Release-Activated $Ca^{2+}$ Channel |
| DMSO | Dimethyl Sulfoxide |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylene Diamine Tetraacetic Acid |
| ER | Endoplasmic Reticulum |
| EtOH | Ethanol |
| G protein | Guanine Nucleotide Binding Protein |

| | |
|---|---|
| GI | Gastrointestinal |
| GPCR | G Protein-Coupled Receptor |
| HEPES | 4-(2-Hydroxyethyl)-1-PiperazineEthaneSulfonic Acid |
| $I_{CRAC}$ | CRAC Current |
| $IP_3$ | Inositol 1,4,5-Trisphosphate |
| $IP_3R$ | Inositol 1,4,5-Trisphosphate Receptor |
| MCU | Mitochondrial Ca2+ Uniporter |
| MgGreen | Magnesium Green |
| MPTP | Mitochondrial Permeability Transition Pore |
| NAADP | Nicotinic Acid Adenine Dinucleotide Phosphate |
| NAD | Nicotinamide Adenine Dinucleotide |
| NCX | $Na^+$ /$Ca^{2+}$ Exchanger |
| PAR2 | Protease Activated Receptor 2 |
| PARS | Protease Activated Receptors |
| PI | Propidium Iodide |
| $PIP_2$ | Phosphatidylinositol Bisphosphate |
| PLC | Phospholipase C |
| PMCA | Plasma Membrane Calcium-Activated ATPase |
| POAEE | Palmitoleic Acid Ethyl Ester |
| PTP | Permeability Transition Pore |
| PZ | Pancreozymin |
| RNS | Reactive Nitrogen Species |
| ROS | Reactive Oxygen Species |
| RyR | Ryanodine Receptor |
| SERCA | Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase |
| SOAR | STIM-Orai Activation Region |
| SOCE | Store Operated Calcium Entry |
| STIM1 | Stromal Interaction Molecule 1 |

| | |
|---|---|
| Tg | Thapsigargin |
| TLC-S | Taurolithocholic Acid 3-Sulfate |
| TPCs | Two-Pore Channels |
| ZGs | Zymogen Granules |
| $\Delta\psi_m$ | Mitochondrial Membrane Potential |

# Chapter 1

# Introduction

### 1.1 Acute Pancreatitis

The term Acute Pancreatitis refers to an acute inflammatory disease of the pancreas involving peripancreatic tissues (1–3). The disease is commonly characterised by abdominal pain and elevated serum pancreatic enzyme levels. The main inducing factor of the disease is the premature activation of digestive enzymes within the pancreas. Despite the pancreas evolving multiple mechanisms to prevent this premature activation, under certain conditions, the enzymes can become activated. This is a major issue which can escalate and cause a lot of damage to the pancreas. As a consequence of this unwanted activation, the surrounding tissue becomes damaged and induces cellular necrosis. This necrosis then triggers a cyclic effect by damaging other surrounding tissue which again, induces necrosis, therefore, worsening the condition of the pancreas and the disease (4–6). In the United States of America per year, the prevalence of acute pancreatitis varies between 13 to 45 cases per 100,000 people whilst the prevalence worldwide ranges between 4.9 and 73.4 cases per 100,000 (7–10). However, acute pancreatitis is one of the most common reasons for gastrointestinal related hospital admissions. Furthermore, the incidence of acute pancreatitis along with its mortality rate are ever increasing.

In the United States alone there were 274,000 hospitalisations due to acute pancreatitis per year in 2006 but increased by 12% in the span of 6 years (11,12). Whilst the mortality rate of Acute Pancreatitis is more than 3000 deaths (1 out of 100,000) per year, the occurrence of acute pancreatitis greatly increases the risk of chronic pancreatitis occurring (13–15). The incidence of chronic pancreatitis is between 5 and 12 people per 100,000 (16). Furthermore, similar to the relationship between acute pancreatitis and chronic pancreatitis, the occurrence of chronic pancreatitis has a significant impact upon patients' chances of developing pancreatic cancer (16–18). This chain of development is extremely worrying as pancreatic cancer itself is the fifth most common cause of cancer death with an extremely low 5-year survival rate ranging from 3-4% (17,18).

During the initiation of acute pancreatitis, various different factors are known to be linked with causing the development of the disease. As shown in Table 1, there are

multiple known causes of acute pancreatitis. The majority of cases are caused by either alcohol or gallstones which normally make up around 70%-80% of incidences (5,13). These two main causes of acute pancreatitis are heavily linked with pathological mechanisms at the cellular level. Although the exact connection between gallstones and acute pancreatitis is not completely understood, the hypothesis includes the blocking of pancreatic ducts by gallstones. This obstruction is thought to cause a reflux of bile into the pancreatic duct and in doing so, injures the surrounding tissue. Although the specifics of this reflux are not known, it has been well documented the pathological consequences of bile acids and alcohol upon pancreatic cells (19,20). These two major inducers of acute pancreatitis trigger multiple complex $Ca^{2+}$ signalling mechanisms which will be explored in detail later, along with their effects upon differing cell types.

Not only is alcohol abuse linked with acute pancreatitis, it is also strongly linked with the incidence of chronic pancreatitis. Acute pancreatitis and chronic pancreatitis go hand in hand, the occurrence of acute pancreatitis greatly increases the risk of chronic pancreatitis developing (21,22). However, the ways in which alcohol is consumed, the risk of developing pancreatitis changes. The excessive consumption of alcohol increases the risk of developing pancreatitis, however, surprisingly less than 10% of heavy drinkers develop pancreatitis, making heavy drinkers a minority of overall pancreatitis cases (21). People who consume 3 or more alcoholic drinks per day during a period of at least 5 years have an increased risk of developing pancreatitis that is 4 times greater than people without a history of alcoholism (16,22,23). Collating this together with the surprisingly low “10% of heavy drinkers develop pancreatitis”, it is hypothesised that alcohol increases the sensitivity of the pancreas to other pathological/acute pancreatitis inducing agents (23).

Further statistics of the incidence of acute pancreatitis also reveals that alcohol-related pancreatitis is much frequent in men than in women. The reason for this, is thought to be related to genetic x-linked differences between the two sexes (24). Genetics also play another role as a factor of pancreatitis occurrence, a number of

different gene mutations are known to affect the pancreas, such as genes which encode the serine protease inhibitor Kazal type 1 (SPINK1), cystic fibrosis transmembrane conductance regulator (CFTR), calcium-sensing receptor (CaSR), chymotrypsin C, claudin-2 and cationic trypsinogen (PRSS1) (25,26). Additionally, as highlighted in Table 1.3 there are other known causes ranging from drug abuse and smoking to hyperlipidaemia and hypercalcemia (13,27–33).

***Table 1.1 Causes of acute pancreatitis***

*Table outlining the different known causes of acute pancreatitis. Modified from Forsmark et al. (192).*

| Known Cause | Approx. Occurrence (%) |
|---|---|
| Gallstones | 40 |
| Alcohol | 30 |
| Hypertriglyceridemia | 2-5 |
| Drugs | <5 |
| Autoimmune | <1 |
| ERCP | 5-10 |
| Trauma | <1 |
| Infection | <1 |
| Surgical Complication | 5-10 |
| Associated Conditions | Common (Diabetes, Obesity and Smoking) |

### 1.2 Acute Pancreatitis is Characterised by Abnormal Calcium Signalling

For years it has been known that calcium signalling is also involved during the initiation of acute pancreatitis within pancreatic exocrine cells. During the development of acute pancreatitis, calcium signals are significantly increased which lead to numerous intracellular effects. Pro-enzymes within Pancreatic Acinar Cells (PACs) can become prematurely activated, disrupting intracellular machinery (34). Excessive intracellular $Ca^{2+}$ concentrations are evoked by combinations of bile acids or fatty acids with alcohol which is not surprising since the principal causes of acute pancreatitis are biliary disease and excessive alcohol consumption (5). However, other causes of acute pancreatitis have now also been demonstrated to induce intracellular calcium overload such as asparaginase, a successful drug used to treat acute lymphoblastic leukaemia.

The excessive release of $Ca^{2+}$ from intracellular stores and the influx of $Ca^{2+}$ from extracellular fluid can create an undesired level of $Ca^{2+}$ within the cytosol. This overload concentration decreases the production of ATP in mitochondria as well as activating proteases. Both of these events then induce necrosis which causes the expulsion of intracellular structures and compounds (35). Consequently, this release of intracellular compounds stimulates local inflammation of the tissue, therefore, inducing acute pancreatitis. Although the induction of acute pancreatitis by calcium overload has been known for some time, there are no feasible therapeutic agents available to effectively combat the ever-increasing mortality associated with acute pancreatitis. In light of this, current and recent attempts have been made to test potential therapeutics by monitoring the compounds effect on intracellular calcium concentrations, specifically aiming to prevent the calcium overload. Furthermore, the pathological cellular effects of acute pancreatitis have primarily been recorded within PACs. However until relatively recently, the role of Pancreatic Stellate Cells (PSCs) in pancreatic physiology and pathology was unclear as the majority of experiments investigating PSCs have been carried out on cultured PSCs (36). The innovative research which has been undertaken at Cardiff University has allowed the study of PSCs within pancreatic lobules (37,38). Despite this, the specific

physiological and pathophysiological roles of PSCs have eluded researchers apart from potentially discovering the role of PSCs in the fibrotic stages of acute pancreatitis which will be discussed later.

### 1.3 Anatomy and Histology of the Pancreas

Historically, the pancreas was often overlooked as an organ of the body. Derived from its Greek name “pan kreas” meaning “all flesh” it was believed by Aristotle that its function was to protect great vessels of the body. It was later claimed by Galen that the pancreas was a cushion for the stomach. Moving forward to the renaissance era, dramatic observations were made of the pancreas by scientists such as Vesalius, Johann Wirsung and Regnier de Graaf. Andreas Vesalius described the pancreas as being a gland impressed with vessels running through it (39). Johann George Wirsung depicted the main duct of the pancreas which was later named after the anatomist.

These observations included the pancreas’ ductal structure and secretion into the intestine. During the 19$^{th}$ century it was recorded by Claude Bernard and others that the pancreatic secretion had digestive properties for compounds such as fat, protein and carbohydrates. The extremely important pancreatic islets were observed by Paul Langerhans in 1869 and by Miring and Minkowski during their study of experimental diabetes shown by in a dog following a pancreatectomy in 1889. Seven years later in 1896, Hans Chiari made the important discovery of pancreatic autodigestion during pancreatitis. With the fundamental understanding of the pancreas’ histology and function, the 20$^{th}$ century involved advanced discoveries including the composition of pancreatic secretion, specific cell types within the pancreas as well as the molecular mechanisms which are involved to carry out the pancreas’ exocrine and endocrine functions.

The pancreas is located transversely behind the stomach, in the left upper abdominal cavity across L1 and L2 of the spine and is divided into 4 constituent sections, the tail, body, neck and head. The largest section of the pancreas, the

body, is situated behind the base of the stomach with the head of the pancreas being embedded in the duodenal loop. Anteriorly located to the super mesenteric artery is the neck of the pancreas. Located in between the neck and tail is the main body of the pancreas which is close to the lumbar region. The tail section is thin, narrow and located towards the spleen. Specific to the tail, the tissue is surrounded by serosa whereas the remainder of the pancreas sections are found within the retroperitoneal space. Due to the size of this space, the lesions of the pancreas are usually found deep within the tissue. Throughout the tissue, there are two main ducts within the pancreas. Firstly, the duct of Wirsung is commonly known as the main pancreatic duct whilst the duct of Santorini is the accessory pancreatic duct. The pancreatic juices pass through the ampulla of Vater and flow through these two ducts to the duodenum.

### 1.4 Multiple Functions of the Pancreas

The pancreas functions both within the endocrine and exocrine systems of the body. However, these two functions are not necessarily carried out by the same cell types. Approximately 1-2% of pancreatic tissue consists of small, clustered areas of endocrine cells which are known to form islets of Langerhans (40). These islets are surrounded by exocrine tissue which is vastly made up of Pancreatic Acinar Cells (PACs). These islets of Langerhans also vary in shape and size throughout the pancreas with larger number of smaller islets located in the head of the pancreas whilst larger islets are more commonly found in the tail section (40,41).

Within these islets of Langerhans, different cell types have been identified which play different roles in the endocrine function of these islets. The majority of these islets are made up of β cells which importantly produces the body's insulin. β cells account for 65-80% of the cells within the islets of Langerhans. Another cell type is α cells which are involved in blood glucose homeostasis (42). These cells secrete glucagon which is a vital hormone within the body's homeostatic hormonal system (43). Glucagon's protection against extreme decreases of blood glucose levels in combination with the secretion of insulin from β cells creates a harmonious system to restore normoglycemia from hypoglycaemic levels (42). Another cell type within

these islet structures are δ cells which have the key function to regulate the anti-hypoglycaemic responses of insulin and glucagon (44). δ cells secrete the hormone somatostatin which inhibits the secretion of insulin and glucagon from β and α cells respectively (44,45). Furthermore, the islets of Langerhans are also the home to Pancreatic Polypeptide (PP) cells which secrete pancreatic polypeptide hormone. This peptide is an integral part in the pancreas-gut-brain axis as well as its inhibitory actions in the gut and regulation of pancreatic secretions. Additionally, at low concentrations of glucose concentrations, pancreatic polypeptide has been found to inhibit glucagon secretions (46–48).

Although numerous different cell types have been identified within the pancreas, there are still more, and more cell types being found. The endogenous ligand of the growth hormone secretagogue receptor, ghrelin, was primarily found to be expressed in the stomach along with the intestine, brain (49) and testis (50,51). However, ghrelin expression has now also been found in the heart (52), lungs (53), immune cells (54) and the pancreas (50). The discovery of ghrelin expression within the pancreas led to the finding of a new islet cell type (50).

The structuring of pancreatic cellular architecture differs vastly between different mammalian species whilst the makeup of cellular islets surprisingly remains relatively similar. In periphery in rodent islets, they are primarily made up of β-cells which are located in the central region of the pancreas whilst being surrounded by other cell types (55). On the other hand, the islet cells found in humans have shown very strong interconnecting signalling between α-and β-cells within the islets which constitutes functional implications for islet cell function (56). In contrast to these endocrine functions of pancreatic cell types, the majority of exocrine functions are carried out by pancreatic acini and ductal cells which are not located within these islet structures. These cells secrete a pancreatic fluid that flow into the duodenum to neutralise stomach acid. This pancreatic secretion is comprised mainly of water and bicarbonate ions with the alkaline bicarbonate ions being the functional compound to neutralise the stomach acid and chime. The neutralisation of these acids is essential to create the most suitable pH conditions for digestive enzymes

which are also released by the pancreas in addition to other enzymes which function within the small intestine. The enzymes secreted by the pancreas are stored and created in PACs. Secretory granules within these cells store enzymes such as trypsinogen, chymotrypsinogen, lipase and amylase. When required, these granules exocytose and expel their enzymatic contents out of the cell, into the pancreatic fluid. This pancreatic fluid then flows through the ductal system of the pancreas until it reaches the duodenum where it is deposited into the small intestine. Some of the enzymes present in the pancreatic fluid are known as precursor enzymes which are not fully functional in their secreted form (57). This is vital for the pancreas to carry out its exocrine function as it prevents the digestive enzymes from digesting tissue and proteins within the pancreas itself. This exocrine function of the pancreas is tightly regulated and controlled by the parasympathetic nervous system but mainly by hormones. Concentrations of regulatory hormones such as secretin, gastrin and cholecystokinin (CCK) are well known to impact the release and production of pancreatic secretion and enzymes. This regulatory control will be looked at in more detail later.

### 1.5 Pancreatic Endocrine Function

The main endocrine function of pancreas is its major role in the regulation of glucose uptake and digestion (58). This important function is facilitated via the release of insulin. Although we have known this for numerous years, the pancreas' association with diabetes has only been discovered during the past 200 years or so. However, the symptoms of diabetes have been recorded throughout history as far back as Egyptian times with manuscripts dating around 1500BC relating to the symptoms of diabetes. However, at the time, people were not able to link these symptoms to the a chronic disease of the pancreas (59). Further accounts in history suggest the improving understanding humanity knew about diabetes. The term diabetes was first coined by a Greek physician, Aretaeus of Cappadocia (2nd century AD). It arises from the Greek verb διαβαινω (diabaino) which means I pass through. This gives way to the nomenclature of diabetes defining the condition of which fluid

runs through the body. It is recognised that Aretaeus produced the earliest and one of the most accurate descriptions of diabetic symptoms in history. His descriptions ranged from extreme thirst and nausea to restlessness and rapid death. His writings of diabetes are remarkable and yet still to this day, it is unclear how Aretaeus was able to make such precise descriptions purely by observations of a disease which is relatively rare (59).

Another important step in the history of the pancreas was in the 17th century where an English physician and anatomist named Thomas Willis coined the term 'diabetes mellitus'. This term indicates that people who had the condition at the time passed sweet tasting urine. Although Willis was the first European medical writer who acknowledged the sweetness of diabetic patient's urine, he was not the first as it was also recorded by an Indian physician, Sushruta, who mentioned this symptom of the disease in the 7th century. Although this rediscovery was important, Willis was still unaware of the pancreas' role in the disease and believed that it was due to the blood and kidneys which was caused by an "an ill manner of living" due to eating habits and psychological status. Despite this, he also identified the neuropathy in diabetic sufferers describing it as "stinging and other…frequent contractions or convulsions". In addition to his observations, Willis suggested dietary changes as a therapeutic strategy which resulted in an improvement of the patient's condition within a month. However, following the patient's recovery, he soon returned to his previous dietary habits. Despite the observations that Willis made, he still could not explain the reason why diabetic patients produce sweet tasting urine (59).

Some two centuries after Willis, the medically trained Claude Bernard produced extremely important research which significantly aided humanity's investigation of diabetes (59). However, Barnard's research did not initially investigate the pancreas. Even so, Barnard's research would revolutionise the thinking of 19th century physicians regarding diabetes. At the time, scientists hypothesised the role of the pancreas in the physiopathology of diabetes as they found stone-filled or atrophic pancreases in diseased patients during post-mortem examinations.

Nevertheless, as these scientists thought that the pancreas was purely an exocrine organ, the post-mortem findings were purely interpreted as chance phenomena. As a consequence, the experimental physiologist, Claude Bernard sought to evaluate this hypothesis by experimenting on a dog. Initially, Bernard incorrectly assumed that diabetes was a “nervous affection of the lung”. Therefore, his experiment started with the injection of grape sugar into the jugular vein of a dog whilst at the same time, extracted blood from the carotid artery. His findings did not show any reduction of sugar contents whilst the blood passed through the lungs. As this finding came as a surprise to Bernard, he decided to continue with multitudes of new experiments involving the feeding of dogs with either carbohydrate-rich or meat only diets in order to identify organs which are responsible for the destruction of glucose. In conclusion of all of his findings, Bernard deduced that the liver was a vital organ in diabetes by storing a water insoluble starchy substance which was converted to sugar/glucose and then secreted into the blood. Bernard named this new substance glycogen and assumed that excessive secretion of glucose from the liver was the cause of diabetes (60). Although Bernard’s conclusion had not solved nor correct regarding the occurrence of diabetes, his research investigating glycogenic action of the liver was vital in the ground-breaking research that soon became gluconeogenesis which significantly promoted the study of diabetes (59).

Following on from these, events in the end of the 19$^{th}$ century proved to be a major turning point in our understanding of diabetes. During this time, Oskar Minkowski and Joseph von Mering had varying discussions regarding the involvement of the pancreas in digestion and absorption of fats. As a consequence, these two scientists decided to perform a pancreatectomy on a dog. Following the operation, the dog remained alive, however, whilst Minkowski observed the animal, he noticed that the dog soon developed polyuria. Following further testing, Minkowski discovered that the urine contained 12% sugar. As this was a surprise to Minkowski, he assumed that the dog had developed diabetes due to the dog being treated with phlorizin by von Mering during other experiments. Therefore, Minkowski repeated the pancreatectomy in three more dogs who had no sugar in their urine prior to the

operation. However, the initial experiment's findings were repeated with all three of the dogs developing glycosuria (59,61,62). To add even more evidence to his findings, Minkowski implanted a small piece of pancreas subcutaneously in these depancreatized dogs. This provided further evidence which supported the inclusion of the pancreas in glucose regulation as the implants prevented hyperglycaemia until removal of the implant or degeneration of the implant (59).

The final major discovery in understanding the principles of pancreatic physiology was a collaborative study which started in 1921. Although at this time it was known the pancreas played a pivotal role in the regulation of glucose, it was not known how. In 1921 Frederick Banting, who worked in the laboratory of Professor John Macleod, started a collaborative effort with a medical student, Charles Best, to investigate the regulatory mechanism involved in glucose regulation (63). Over a period of weeks, these two physicians ligated pancreatic ducts from dogs which eventually led to the removal of the dogs' degenerated pancreata. The dissected tissue was later crushed, frozen and then dissolved into saline solutions. These new solutions were then intravenously injected into depancreatized dogs to test the effects of this pancreatic solutions. The results showed a remarkable drop in the dogs' blood sugars within two hours of administration. Following these results, further experiments using different methods of administration as well as foetal calf pancreata were utilised but all demonstrated similar findings (59). Towards the end of 1921, a chemist named James Collip teamed with Banting and Best who all collaboratively developed a new method of extraction and purification of the substance obtained from the pancreas. This new substance was named insletin which was later turned into insulin by John MacLeod.

To test this newly isolated substance even further, the group decided to test it in humans. On the 11$^{th}$ of January 1922, a 14-year-old boy named Leonard Thompson was being treated for diabetes in Toronto Hospital. Following previous treatments, the boy's condition was worsening and delved into a critical state. At this point, he received 15mL of insulin however, he developed abscesses at the injection site and his condition worsened. Collip then produced a further improved quality of isolated

insulin which Thompson received on the 23rd of January (63). Following his second injection, Thompson showed signs of recovery with impressive results being shown as blood glucose levels fell from 520 mg/dL to 120 mg/dL in just 24 hours. Furthermore, urinary ketones were also abolished during this time. For 13 years, Thompson continued the treatment he was given by Banting, Best and Collip until his death at the age of 27 due to pneumonia (59). The pioneering work that was carried out by this team was revolutionary in the fight against diabetes. It saved millions of lives with diabetic patients living mostly normal lives. Lilly Pharmaceutical Company collaborated with the group and produced the first commercially available insulin product, Iletin in 1923. This work was so important, it was recognised at the highest level with a Nobel Prize being awarded to Banting and MacLeod for the discovery of insulin. However, this was felt by many to be unfair including Banting who thought the prize should have been shared between himself and Best. As a consequence, Banting shared his cash prize with Best whist in turn, MacLeod shared his prize with Collip (59).

### 1.6 Pancreatic Exocrine Function

Alongside the lifesaving work which lead to the discovery of diabetes and its treatment, vital research was undertaken which vastly increased mankind's understanding of the exocrine function of the pancreas. The Dutch physician and anatomist, Regnier de Graaf investigated the secretory juice of the pancreas in the 17th Century. He did this by using a hollow quill of a goose feather and manually canulating the pancreatic duct of a dog (63). However, the theories that de Graaf created following the extraction of pancreatic juices were vastly incorrect as it wasn't until a century later when Claude Bernard demonstrated that pancreatic juices could emulsify fatty foods (64). Not only did Bernard provide some of the core principal understandings of the endocrine functions of the pancreas but also initiated the thinking that perhaps the pancreas also yielded some exocrine functions (59). He demonstrated to the world that the pancreas was clearly involved in digestion by observing the emulsification and absorption of fatty foods as well as

being able to convert starch into sugar. During the time of Bernard, the general consensus was that body's digestion was entirely contained within the stomach. Therefore, Bernard's findings truly revolutionised our thinking about the pancreas and digestion.

The next major breakthroughs in discovering the exocrine function of the pancreas were made during the 19th and 20th century. During the 19th century the first physiologist who won a Nobel Prize, Ivan Petrovich Pavlov, was awarded the prize for his research on the neural control of salivary, gastric, and pancreatic secretion. Pavlov's research demonstrated control of pancreatic secretion and showed its mediation by neural reflexes via unique pancreatic fistula (65). However, Pavlov's studies focused very much on purely neural control of pancreatic secretion. Ernest Starling and William Bayliss disputed Pavlov's results as they were unable to replicate Pavlov's findings at the time. As a consequence, Starling and Bayliss initially proposed hormonal control was most important in the regulation and secretion of pancreatic juices. This led to Starling and Bayliss formulating an exciting new experiment involving an anaesthetised dog which had its nerves serving the jejunum dissected and removed. This resulted in the jejunum only being connected to the rest of the body via blood vessels. The introduction of HCl into the duodenum of the dog resulted in a secretion produced from the pancreas following several minutes (66). The results of this experiment clearly show the humoral mechanism involved in pancreatic secretion. The two isolated this chemical and termed it secretin (67).

When Pavlov's team repeated the experiments of Starling and Bayliss it was recorded that they watched the experiment with bated breath and in silence. Once the results were clear, Pavlov walked into his study and returned half an hour later famously saying; "*Of course they are right. It is clear that we did not take out an exclusive patent for the discovery of the truth*" (66). Pavlov then moved his research to focus more on conditioned reflexes and conditioning. Following the conclusions of all of these experiments it was coincidentally found that the reason for Starling and Bayliss's inability to repeat Pavlov's initial findings was due to the pre-

anaesthetic morphine treatment that they used at the time (68). Starling later recognised this failure and conceded that dual, hormonal and neural systems control pancreatic secretions (66). Up until this day, secretin is recognised as the key pathway which regulates pancreatic secretion via a negative feedback mechanism. Acidic chyme released from the stomach initiates the production and release of secretin from small intestinal mucosal S cells. This in turn stimulates the release of pancreatic juices into the duodenum consequently neutralising the acidic chyme as it enters the small intestine (66).

In the years following the major breakthrough in control of pancreatic secretions, many new hormones were discovered which all have a role in secretory control. The second hormone that was discovered to be able to stimulate the release of pancreatic secretions was pancreozymin by Harper and Raper in 1943 (69). Whilst working at Manchester University, the two physiologists were able to record several key elements about pancreozymin control. By extracting pancreozymin from dogs, pigs and cats and then intravenously injecting this substance into cats showed an increased secretion of pancreatic enzymes whilst having no effect on the volume of pancreatic juice secreted. They also demonstrated several properties of this new hormone which included thermostability, stability in acidic solutions but not in alkali, denatured by pancreatic juice, and its distribution being similar to that of secretin within the small intestine with no presence in the gastric mucosa (69).

Following these events, Erik Jorpes and Viktor Mutt discovered the hormone cholecystokinin (CCK) and found that this new hormone showed similar biological properties to pancreozymin. Both hormones stimulated pancreatic enzyme secretion in similar methods (70). Since Jorpes' and Mutt's discovery there were questions posed whether CCK and pancreozymin were the same hormone. The observed effects of CCK and pancreozymin have both been recognized from being stimulated by one protein. Therefore, it is generally known as CCK which fulfils both the release of enzymes from the pancreas and the force which pushes bile into the duodenum by causing contraction of the gallbladder. However, it is agreed that

pancreatic secretion is controlled by neurohormonal regulation via a complex biology of multiple different receptors and signalling pathways (40,71).

The main exocrine functional part of the pancreas is the combination of the acini and ductules. The ducts of the pancreas flow throughout the organ getting smaller and smaller. These ducts are surrounded by PACs which make up the majority of the pancreas. Within these structures are also Centro Acinar Cells (CACs) which help makeup the composition of the ductal interface as shown in Fig. 1.1. These cells are extremely important by allowing the pancreas to fulfil its exocrine role. CACs regulate the contents of the ductal lumen by secreting carbonic anhydrase II or bicarbonate ($HCO_3$) into the pancreatic fluid. This enables the pancreatic fluid to become alkaline and in doing so neutralises the stomach acid when it reaches the duodenum (72,73). At the end of these ducts are small clusters of PACs. Hundreds of these cells are packed tightly in organised clusters named acinus. This complex organisation of cells further shows how detailed the modelling of pancreatic tissue is, even still, these cells communicate with each other via both direct and indirect chemical and electrical signalling via gap junctions (74,75). The culmination of these regulatory mechanisms is to fulfil the pancreas' role of producing and secreting digestive enzymes.

There are multiple different enzymes and proenzymes which are produced and secreted from the pancreas shown in Table 1.2. The process of enzyme production is vastly similar to that of protein production within the PACs. This process involves 6 successive stages starting from the synthesis of the enzyme to its discharge into the pancreatic juice and duodenum (76). This is a complex process which includes the entirety of the PAC's intracellular machinery. However, key studies that have been carried out over the last 50 years demonstrated the first instances of neurohormonal control of pancreatic regulation alongside calcium coupling (77). These experiments not only demonstrated the involvement of calcium within the regulation of pancreatic processes but also the process known as "stimulus-secretion coupling". This process was shown to regulate the secretory process of exocytosis through changes in cytosolic $Ca^{2+}$ concentrations. Furthermore, this

regulation via intracellular $Ca^{2+}$ concentrations enabled the use of extremely small levels of initial stimuli as this initial trigger becomes massively amplified during this process of stimulus-secretion coupling. The amplified signal mediated by multiple messenger compounds along a long intracellular chain reaction leads to the ultimate stimulation of the cell, therefore, stimulating the release of digestion enzymes (78–82). As previously mentioned, the hormone CCK is mainly responsible for triggering and regulating the pancreas. Both the release of enzymes and juice are a result of fluctuating intracellular calcium levels in CACs and PACs which is stimulated by extracellular CCK concentrations (82).

***Table 1.2 Digestive proenzymes alongside their active enzyme equivalents found within the pancreas.***

*Table highlighting the proenzymes produced by the pancreas and their respective activated variants. Table adapted from Pandol et al. (83–86).*

| **Proenzymes** | **Enzymes** |
|---|---|
| Trypsinogens (1,2,3) | |
| Chymoptrypsinogen (A, B) | α-Amylase |
| Procarboxypeptidase A (1, 2) | Lipase |
| Procarboxypeptidase B (1, 2) | DNase |
| Prophospholipase (I, II) | RNase |
| Proelastase | |
| Mesotrypsin | |

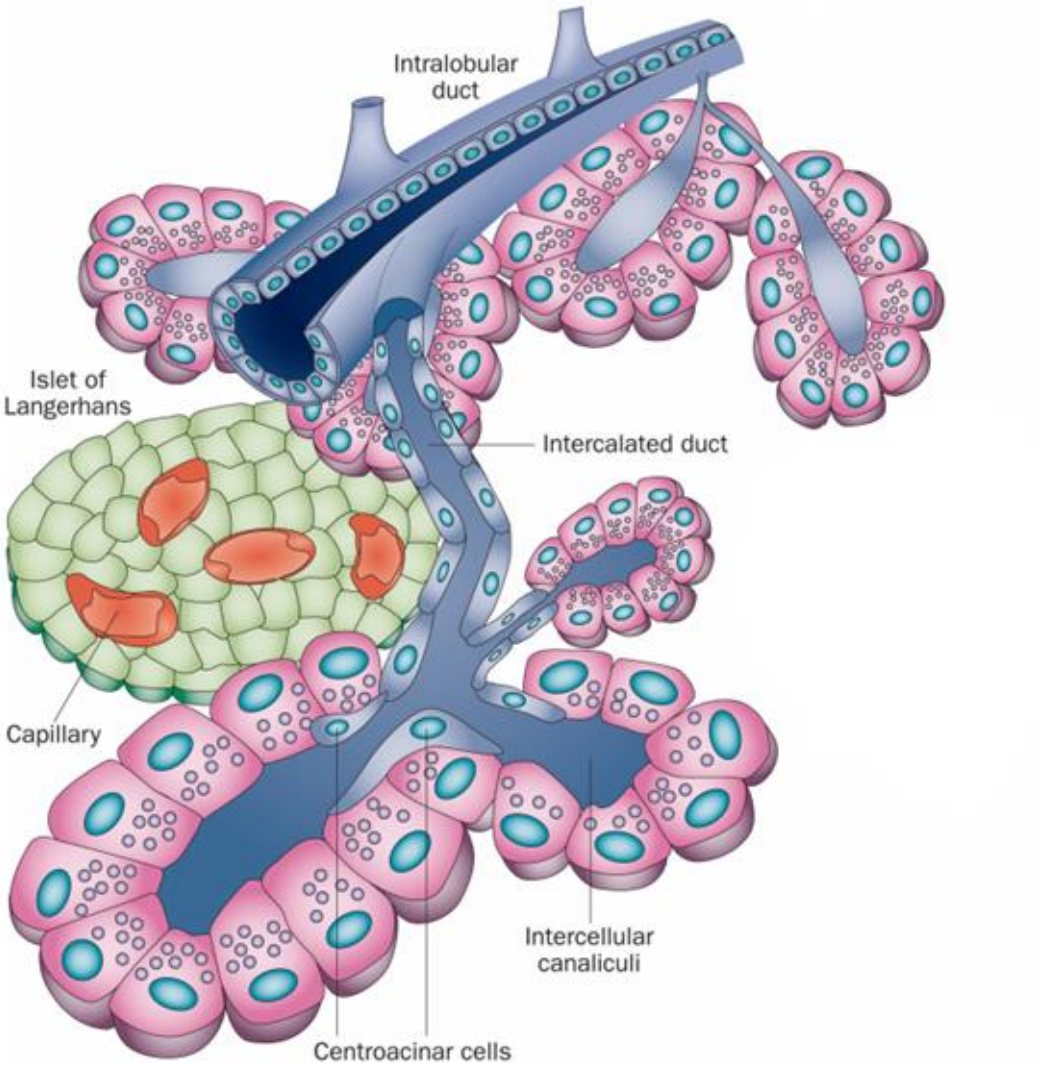



***Figure 1.1 The exocrine organisation of the pancreas. The functional unit of the exocrine pancreas is composed of an acinus and its draining ductule.***

*An acinus surrounding a central lumen open to the duct system is formed by pancreatic acinar cells. Pancreatic acinar cells synthesize, store and secrete digestive enzymes for the digestion and absorption of food in the small intestine. Digestive enzymes are secreted through the apical membrane of the acinar cell into small, intercalated ducts that are directly connected to increasingly larger intralobular ducts that join the main pancreatic duct (44). Modified from Logsdon & Ji (87).*

### 1.7 Pancreatic Acinar Cellular Structure

The exocrine function of the pancreas is clearly its primary objective, therefore, the vast majority of the pancreas is made up of PACs. According to *Bolender* (88), approximately 82% of the pancreas consists of PACs. Furthermore, analysis of the structure within PACs reveals that the cells heavily consist of Rough Endoplasmic Reticulum (RER), mitochondria and zymogen granules. However, what is most interesting about the intracellular structure of PACs is the distribution of the membrane surface area in relation to its organelles. Although 22% of the cellular volume is RER it was found that 60% of the cellular membrane was in contact with the RER, clearly demonstrating its key role to play in the operation of PACs (88).

The intracellular layout of PACs seems to clearly show two main regions as demonstrated in Fig. 1.2. These two regions are the basolateral and apical poles of the cell which seems to divide the cell. The two poles of the cell seem to be distinguished by the mitochondrial belt which further acts as a calcium buffer between the poles. This mitochondrial buffer belt can moderate calcium from the apical pole to the basolateral. The basolateral pole of the cell is far greater than the size of the apical pole, with up to 90% of the cell being contained within the basolateral pole. Within this basolateral pole is the majority of the Endoplasmic Reticulum (ER) however, the ER has also been shown to penetrate into the mitochondrial belt of the cell (89).

As previously mentioned, the main exocrine function of the pancreas and PACs is to produce and secrete digestive enzymes and proenzymes. These are stored within PACs inside zymogen granules which are located at the apical pole of the cell. The mechanism of secretion is mediated by the hormone cholecystokinin (CCK) and the neurotransmitter acetylcholine (ACh) which is released locally from the vagus nerve. These two compounds both stimulate and regulate the secretory process which takes place via exocytosis of zymogen granules. These granules fuse with the apical membrane of the cell which releases the zymogens into the luminal ducts of the pancreas. The released granules are suspended and transported in a chloride and bicarbonate rich fluid along the ductal system towards the gut. Within the granules

and the isotonic pancreatic acinar juice are a multitude of digestive enzymes and precursor enzymes as displayed in Table 1.2. This pancreatic juice contains both active enzymes and inactive precursors in order to protect itself from internal digestion. The safe active enzymes include α-amylase, lipases and colipase in addition to others, however, it is unable to secrete the active forms of the precursors trypsinogen, chymotrypsinogen and procarboxypeptidases (84,85,90). This is in order to prevent autodigestion of the surrounding tissue within the pancreas (86,91). Instead, the pancreas uses a mechanism which activates the precursor enzymes when they enter the small intestine. This is initiated by enterokinases found in the small intestine which firstly, cleaves Lys-IIe bonds and clusters of lysine from trypsinogen (86). Secondly, binding to the $Asp_4$ group also occurs which dramatically increases the efficiency of the initial cleaving stage. Overall, these two steps are the reason for the incredible specificity of enterokinases to trypsinogen. Furthermore, this activation process in enhanced by the optimal pH of 6-9 in the entrance of the duodenum with ever increasing efficiency when in the presence of low concentrations of bile acids (86). As a consequence of this process, enterokinase converts trypsinogen to its active form 15 trypsin. This whole process is dependent on the cleavage of trypsinogen as the other precursor enzymes are further cleaved by the activated form, trypsin. Trypsin activates all the other precursors, forming their active states, therefore allowing for the digestion of nutrients (86).

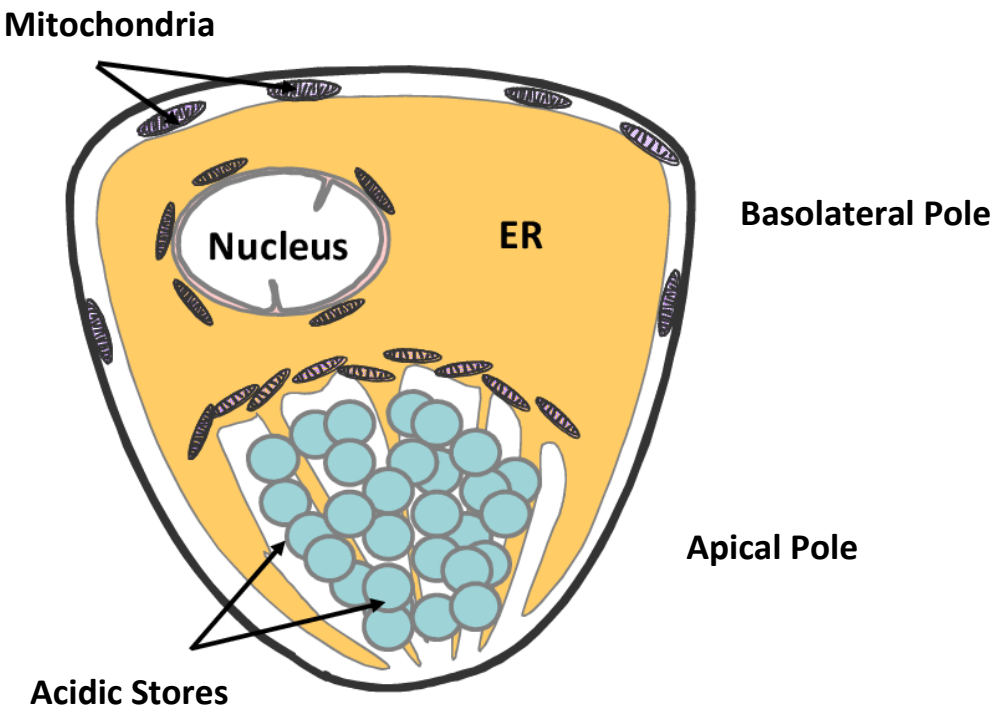



***Figure 1.2. Schematic illustration highlighting the intracellular structure of PACs.*** *Demonstrating the basolateral and apical poles of PACs in addition to the intracellular layout and position of mitochondria, nucleus, ER and acidic stores. Modified from Ferdek* (92)**.**

The pancreatic juice that transports these precursors and enzymes is neutral but mixes with bicarbonate secretions from centroacinar cells to form the medium which neutralises acidic stomach chyme. As previously discussed, the secretion from centroacinar cells is stimulated by the hormone secretin (85,93–95). This stimulation triggers functional physiological mechanisms which are controlled by calcium (96). Within the cell different calcium fluxes are triggered depending on the initial stimulus and whether it triggers physiological or pathophysiological signalling. Not only does the stimulus dictate the signalling outcome but it has been shown that the pattern of cytosolic calcium fluxes also dictates which signalling pathway is triggered (97,98). Furthermore, these patterns of calcium signalling can occur within certain locations within the cell which also affect the resulting signalling pathway (96–98). Following stimulation from normal physiological concentrations of ACh and CCK, small oscillatory intracellular cytosolic $Ca^{2+}$ signals can be triggered which are normally located within the apical pole. These signals do not occur within the basolateral pole of the cell unless there is a global calcium response in the cell which also originates from the apical pole but expands towards the basolateral region (96–98).

Although there may not be significant physiological calcium signalling within the basolateral pole of the cell, there are numerous ACh and CCK receptors within the basolateral membrane. When these receptors are activated, a process follows which produces a secondary messenger response within the cell. These secondary messenger compounds then go on to initiate release of calcium from intracellular and extracellular stores (99–101). During early experiments and studies, investigations into the release of calcium within the cell and its effect on the release of amylase showed the main intracellular calcium store was the ER (77,102,103). These innovative experiments not only identified the ER as being the primary intracellular calcium store but also highlighted the increased efflux of intracellular calcium from intracellular stores in response to ACh and CCK. Additionally, this calcium efflux was also shown to increase the secretion of amylase from PACs (77,102,103). Following on from these foundation-laying studies, it was still unclear

which secondary messenger molecules were responsible for the release of intracellular calcium until some studies suggested that Inositol 1,4,5-trisPhosphate ($IP_3$) was responsible (104). It was discovered that increases in $IP_3$ concentration elicited the mobilisation of internal calcium which proposed $IP_3$ as fulfilling the intermediary role between ACh and intracellular calcium efflux (104,105). Despite this breakthrough at the time, it was still unclear as to why physiological $Ca^{2+}$ signalling in PACs did not occur within the basolateral area of the cell but were consigned to the apical pole which contains little amounts of ER compared to the basolateral region (98,106–109). To aid in our understanding of why this is, experiments using focused $Ca^{2+}$ tunnelling demonstrated the uptake of $Ca^{2+}$ at the base of the cell and into the lumen of the ER (110,111). This ER then extended throughout the cell and eventually leads to the granular apical pole of the cell. This vast ER network revealed projections of the ER deep into the apical pole and in between zymogen granules. This finding posed even more questions as to the role this ER network could play in $Ca^{2+}$ buffering and the transmission of information within the cell via $Ca^{2+}$ signalling (108–111). With this in mind, it was evident that $Ca^{2+}$ uptake at the base of the cell was able to be transported directly to the apex of ER extensions surrounding zymogen granules via the ER.

Not only does $IP_3$ initiate $Ca^{2+}$ release from the ER extensions to transmit cellular instructions to zymogen granules, but the release of $Ca^{2+}$ itself amplifies the signal. Extremely localised, specific signalling occurs within hotspot areas between ER extensions and zymogen granules which consists of $Ca^{2+}$ released from the ER being taken up by zymogen granules. The initial signal of $IP_3$ further induces the recirculation of $Ca^{2+}$ from zymogen granules into the surrounding cytosol as shown in Fig. 1.3, which highlights these $Ca^{2+}$ hotspots (110,111). Furthermore, stressing the importance of $IP_3$ signalling in these areas is the observation of the highest concentration of sensitive $IP_3$ Receptors ($IP_3Rs$) within the apical pole of PACs (112,113). This $Ca^{2+}$ signalling that occurs only in the apical pole of the cell is vital for the activation of $Cl^-$ channels. These $Ca^{2+}$ activated $Cl^-$ channels are tightly regulated by intracellular $Ca^{2+}$ concentrations and are exclusively expressed in the

apical membrane of the cell. Furthermore, these $Cl^-$ channels are critical for the regulation of acinar fluid secretion and enzyme secretion, therefore, the localised acetylcholine-elicited $Ca^{2+}$ signalling controls fluid and enzyme secretion (114,115).

The distribution of the mitochondria within PACs is also just as important as the ER. As previously mentioned, there is a mitochondrial belt spanning the cell which divides the apical and basolateral poles (89,116). This specific distribution of mitochondria prevents the unwanted diffusion of $Ca^{2+}$ from the apical pole to the basolateral pole. In functioning mitochondria, $Ca^{2+}$ released into the cytosol during physiological signalling is absorbed, therefore, the mitochondria act as a $Ca^{2+}$ buffer between the two poles (116,117). As a consequence, when mitochondria function is impaired, the localised apical $Ca^{2+}$ quickly transforms into a global $Ca^{2+}$ response (116,117).

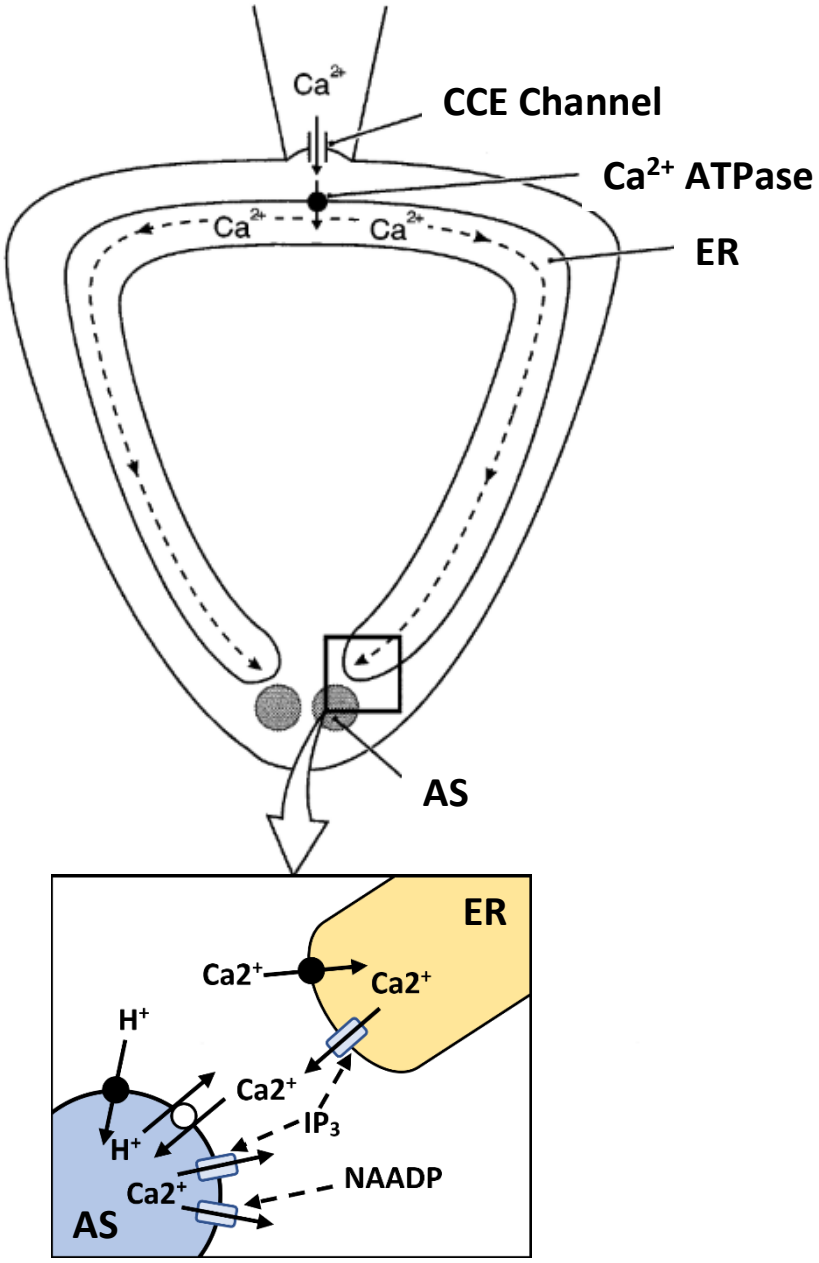



***Figure 1.3 Schematic diagram illustrating $Ca^{2+}$ tunnel from the basal to apical part of the cell with signalling events occurring in the secretary pole.***

*Illustration of the tunnelling effect of the endoplasmic reticulum within PACs. This tunnelling allows for the rapid transduction of signalling events between the basal and apical regions of the cell at which, calcium is released from the endoplasmic reticulum and induces localised calcium signalling surrounding the acidic stores. Diagram modified from Mogami et al.* (111).

### 1.8 Calcium Signalling in the Exocrine Pancreas

Calcium ions are one of the most crucial molecules in physiology. $Ca^{2+}$ is known to play an integral part within signal transduction pathways during both pathological and physiological processes throughout the body (118–120). It is involved in the transduction of external stimuli in regulatory process during but not limited to muscle contraction, fertilisation, cardiac rhythm and enzyme secretion (121–124). This crucial signalling molecule is also significantly involved in cellular life-cycle processes such as apoptosis, necrosis, proliferation and gene expression (122,124–126). Furthermore, calcium ions are an integral part of neuronal signal encoding and plasticity which enables the use of $Ca^{2+}$ during neurogenesis, learning and memory formation (122,124,126–130). As a consequence of $Ca^{2+}$ being a key part of so many processes, it is logical that when there is an impairment or malfunction of these processes then pathological consequences ensue such as cancer, neuronal development defects and many others including pancreatitis (123,124,126,128,131).

Physiologically within the pancreas, calcium signalling is majorly involved in the secretion of pancreatic digestive pro-enzymes in PACs. This important role of calcium signalling within PACs has been well documented for several years (13). However, multiple studies have highlighted specific mechanisms of action via $IP_3$ and Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) pathways. Release of $Ca^{2+}$ from the endoplasmic reticulum and $Ca^{2+}$ influxes into the cell induce the secretion of pro-enzymes into the acinar lumen (77). This exocrine function of PACs is monitored and controlled by multiple different regulatory hormones such as insulin, systemic hormones and growth factors (132). However, calcium signalling within PACs are heavily controlled by neurohormonal factors such as Cholecystokinin and Acetylcholine. The complementation of receptors and hormones in signalling enzymatic secretion has been thoroughly discussed and investigated in past studies but the latest research is primarily focussing upon ligand-receptor binding and signal transduction mechanisms which are thought to be heavily involved (132).

### 1.9 Intracellular Calcium Signalling in Pancreatic Cells

Calcium ions are immensely important and are a vital part to intracellular signalling. Within the workings of cells, particularly pancreatic cells, calcium is utilised as an intracellular messaging molecule which regulates cellular and organellular functions. However, there is a vast network of regulatory mechanisms which control the complexities of calcium signalling. The use of calcium signalling within the cell enables it to respond to a multitude of different signalling networks from other cells and organs in the body. These differing signal types are able to influence the desired physiological outcomes on specific cells by altering the intensity of the calcium signal transduced within the cell. The folding and expression of proteins and enzymes are reliant on the levels of intracellular calcium (133). Variations of the intracellular calcium levels can have a resounding effect on the conformations of enzymes, therefore, altering their functionality. There are multiple pathways in which the intracellular level of calcium can change. Firstly, calcium ions can migrate directly into the cytosol of the cell from extracellular stores via activated ion transporters and channels. This influx of calcium will be discussed in more depth later. The other second main pathway of signal transduction is the use of calcium ions as a secondary messenger. This is brought about as a key part of these signal transductions is the interaction of calcium with G protein-coupled receptors (GPCRs) on the cellular membrane (133–135). A signalling cascade initiated by the activation of GPCRs can induce further activation of calcium signalling.

All eukaryotic cells express GPCRs which is also the largest receptor molecule superfamily present on the surface of the cell membrane (136,137). This superfamily is made up of over 2,000 genes which express different subunits and structures which make up GPCRs, also known as seven-transmembrane domain receptors (133,135–137). GPCRs are complex heterotrimeric structures which are comprised of 3 different subunits known as alpha (α), beta (β) and gamma (γ) subunits (138). Due to the vast expression of GPCRs across eukaryotic cells, it is no surprise that there are numerous diseases which are linked to GPCRs (139). As a consequence, GCPRs are commonly specific targets for drug action. More than 50%

of drugs target 4 gene families expressed in cells including nuclear receptors, ligand-gated ion channels, voltage-gated ion channels and class I GPCRs. The majority of these proteins are localised to the cell surface and yet only approximately 22% of all proteins coded in the human genome are located at the cell surface. Nevertheless, almost 60% of all drug targets are present within the cell membrane (139).

Cells' reliance on intracellular calcium signalling requires extremely precise maintenance of calcium homeostasis. As the movement of calcium ions across membranes is vital, both the cellular membrane and organelle membranes, cells create a resting gradient of calcium across each membrane as shown in Fig 1.4 (120). At resting concentrations, there is an approximate 10,000-fold difference in the concentration of cytosolic calcium (100 nM) and that of extracellular stores (10 mM) (120,140). Furthermore, there is also a massive gradient difference between intracellular calcium stores such as the endoplasmic reticulum which harbours a concentration of 100 mM (140). In order to maintain these concentration gradients there are a multitude of key calcium channels, pumps and exchangers which regulate and restrict the movement of calcium ions across membranes. Plasma Membrane $Ca^{2+}$ ATPase (PMCA) pumps clear cytosolic calcium through the cell membrane and into the extracellular space whereas sarcoendoplasmic Reticulum $Ca^{2+}$ ATPase (SERCA) pumps direct cytosolic calcium into intracellular stores, such as the ER and SER (140,141). Both these calcium pumps expend energy stored within ATP to move $Ca^{2+}$ ions across the membrane. PMCA moves one $Ca^{2+}$ ion per ATP molecule hydrolysed whereas SERCA transports two $Ca^{2+}$ ions per ATP molecule (140,141). Another important membrane protein which cooperates with PMCA pumps to maintain the calcium ion gradient across the cellular membrane is the $Na^{+}/Ca^{2+}$ exchanger (NCX) (141–144). This is an antiporter ion exchanger which is also expressed in almost all eukaryotic cells (144). However, although PMCA pumps and NCX fulfil the same outcome, the two proteins have different functional properties which differentiate the two channels. NCX antiporters do not utilise ATP as an energy source, instead, it uses the concentration gradient of $Na^{+}$ across the membrane as a store of energy. Subsequently, NCX exports one $Ca^{2+}$ ion out of the

cell whilst simultaneously importing three $Na^+$ ions (142,144). Furthermore, PMCA pumps have a lower capacity to transport $Ca^{2+}$ ions but have a much greater affinity, whereas NCXs have a low affinity for $Ca^{2+}$ transport but can extrude a much greater number of $Ca^{2+}$ ions in a short period of time (141,142,144). This difference in functionality allows NCXs to become much more useful in excitable cells such as cardiac myocytes and neurons where the generation of action potentials and cellular repolarisation in short timespans is vital (125,141,144–147). As a consequence, NCXs have a much lesser role in calcium signalling within PACs compared to PMCA pumps (148,149). Additionally, the PMCA pumps' functional rate is regulated by a calcium-binding protein named Calmodulin (125,150,151). This protein can significantly affect the pump rate and the binding affinity of PMCA pumps for $Ca^{2+}$ ions (125,150,151).

The control of calcium ions across the cellular membrane are also influenced by voltage-activated $Ca^{2+}$ channels. However, these channels are more commonly found in excitable cells (152,153). These channels are extremely important during the process of exocytosis within excitable cells. During this process in excitable cells, the depolarisation of the membrane leads to the opening of voltage-gated calcium channels which allows a massive influx of calcium into the cell, therefore raising intracellular calcium concentrations (152–156). The sudden increase and spike in intracellular calcium levels is commonly used to initiate exocytosis within cells such as PACs. However, PACs are unable to become electrically excited, therefore, a different mechanism of events is stimulated when exocytosis is required (155,156). Instead, calcium ions are released from intracellular stores which lead to stimulus-secretion coupling events (155,157). As previously discussed, it is agreed that both ACh and CCK stimulate the initial secretory response of PACs. Furthermore, this initialisation is known to be independent of extracellular $Ca^{2+}$ ions, however, the presence of extracellular calcium is crucial for sustained secretory responses (78–82). Following on from the release of calcium from intracellular stores, the increased cytosolic concentration needs to be restored to normal low levels. Therefore, it was inevitably shown that PMCA pumps transported calcium to the

extracellular fluid in order to restore homeostatic levels (158). Furthermore, it was definitively concluded that NCXs were not responsible for this extrusion of calcium in PACs (158). During these cycles of transient and sustained cytosolic calcium signalling triggering exocytosis, another process is initiated to increase cytosolic calcium levels (159–161). This process is Store-Operated $Ca^{2+}$ Entry (SOCE) and is triggered in the event of a reduction of calcium in intracellular stores, therefore, when calcium is released from stores, SOCE is initiated (159–161). SOCE involves the activation of store-operated calcium channels to open in the cell membrane which releases the flow of $Ca^{2+}$ ions into the cytosol from extracellular spaces (159–161). The activation of store-operated calcium channels is in response to increased cytosolic calcium levels which is sensed by modulating proteins such as STIM and Orai proteins (160). These calcium sensing proteins detect the depletion of $Ca^{2+}$ in the ER which triggers a multitude of channels including $Ca^{2+}$ Release Activated $Ca^{2+}$ (CRAC) channels which prolong the sustained secretory stimulation in PACs (159–164).

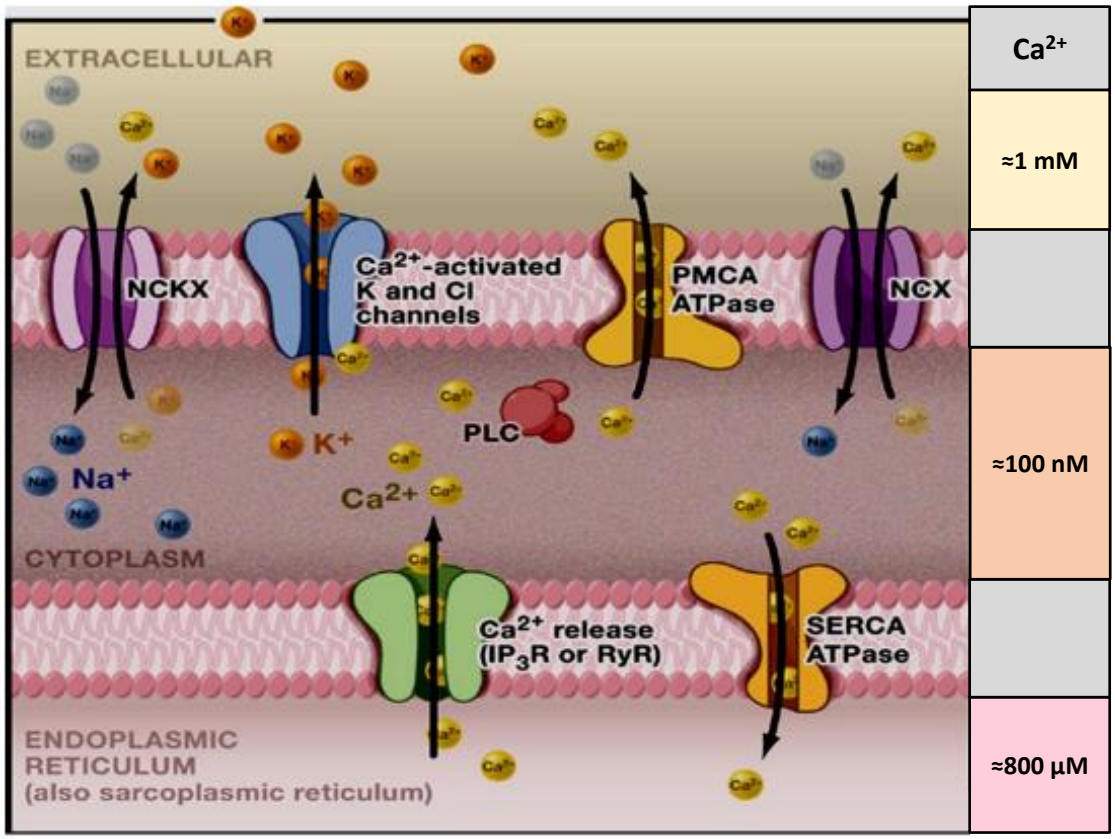



***Figure 1.4 Movement of calcium ions across the plasma and endoplasmic reticulum membranes.***

*Ions including calcium are constantly transported across the membranes within the cell in order to maintain homeostasis. This results in constant concentrations of $Ca^{2+}$ within the cytosol, endoplasmic reticulum and extracellularly. There are two primary extrusion transporters, the sarco-/endoplasmic reticular $Ca^{2+}$ ATPase (SERCA) and the plasma membrane $Ca^{2+}$ ATPase (PMCA). These two transporters extrude calcium ions out of the cytosol by utilising an ATP molecule. Contributing to this extrusion, the $Na^{+}/Ca^{2+}$ exchanger (NCX) imports three sodium ions into the cytosol whilst extruding a calcium ion out of the cytosol. All of these channels, although NCX to a lesser extent, counteract the calcium entry and release into the cytosol via CRAC and $IP_3$/NAADP mediated calcium release. Adapted from Clapham (120).*

### 1.10 Calcium Release in Pancreatic Acinar Cells

The main exocrine function of PACs, like many other cell types, relies heavily on intracellular signalling in response to an initial stimulus such as ACh or CCK (88,99,116,165,166). For some time it has been well established that stimuli such as ACh or CCK initiate repetitive local spiking of $Ca^{2+}$ in specific areas within the cytosol of the cell in order to facilitate the release and exocytosis of zymogen granules (75,108,109,111,155,156,167–169). As previously mentioned, these spiking events of intracellular $Ca^{2+}$ concentrations are a culmination of multiple pathways working in unison and disharmony to either increase or decrease cytosolic levels of calcium when required. These apically localised oscillations are triggered by the hormone CCK or neurotransmitter ACh when normal, physiological concentrations are applied to PACs. There is a delicate balance of feedback mechanisms which maintain these localised signals in the apical region of the cell. If this balance is broken or impaired then global calcium responses are likely which also propagate towards the basolateral pole (35,75,108,109,111,117,155,156,167–172). These global calcium responses are often linked to pancreatic pathology which will be reviewed later.

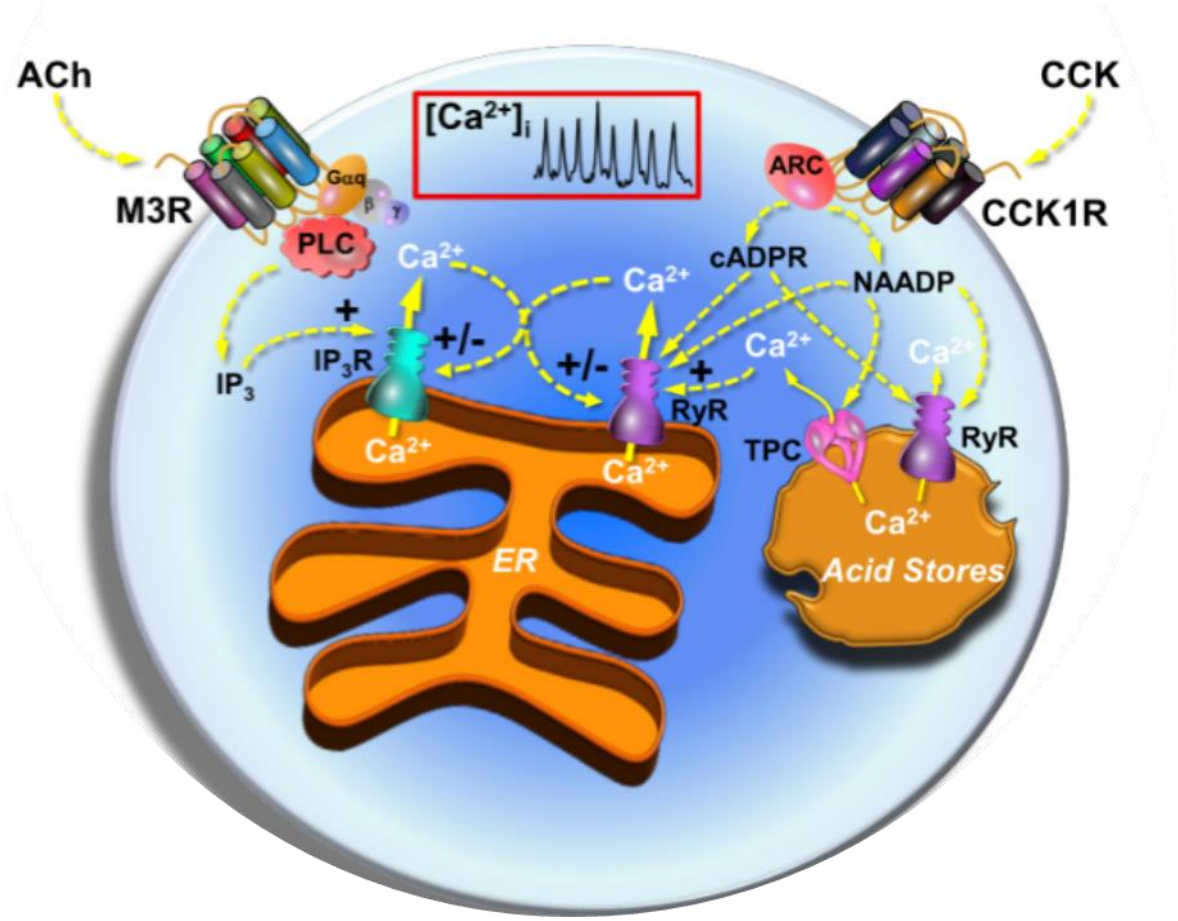



***Figure 1.5 Local oscillatory NAADP- and $IP_3$ mediated calcium signalling events within PACs.***

*The initial induction of signalling occurring at the plasma membrane is commonly triggered by either CCK or ACh as previously discussed. CCK interaction with its CCK1 receptor generates the production of cADPR and NAADP via the ADP-Ribosyl Cyclase enzyme. This generated cADPR and NAADP induce the release of $Ca^{2+}$ from intracellular stores such as the ER and acidic lysosomes via the activation of TPC channels and RyRs. The activation of Muscarinic 3 Receptors by Ach binding triggers the activation of PLC which in-turn produces the signalling messenger $IP_3$. This $IP_3$ then binds to its $IP_3$Rs upon the ER membrane which also release calcium ions into the cytosol. Modified from Petersen et al. (35).*

The stimulation of local apical spiking is initiated via two partly separate pathways depending on whether ACh or CCK triggered the signal. These physiological stimulants trigger different signal transduction pathways despite both stimulants acting upon the cell membrane surface (75,100,101,167,168,173). Furthermore, both stimulants initialise the release of calcium ions from intracellular stores which triggers the creation of localised calcium spiking. This initial release of calcium is primarily regulated via either NAADP or $IP_3$ mediated pathways which are stimulated depending on the stimulant. As demonstrated in Fig. 1.5, ACh binding triggers the release of calcium via $IP_3$ signalling pathway whereas CCK induced calcium release is mediated by NADDP and Cyclic Adenosine Diphosphate-Ribose (cADPR) signalling (89,98,99,123,126,153,167,171,174–177).

### 1.11 $IP_3$ Mediated Calcium Release in PACs

The initiation of $IP_3$ mediated calcium release starts with the binding of ACh and muscarinic receptors on the surface of the cell (173,178). The signalling in PACs is reliant on M1 and M3 muscarinic receptors, however, M3 is much more important due to its much greater affinity to low concentrations of ACh which is normally responsible for physiological signalling (178).

Upon binding of ACh and it's receptor, the subunits of the GPCR conformationally change and in doing so, activate phospholipase C (PLC) (35,161,173,178). This activated PLC then cleaves the phospholipid, Phosphatidylinositol 4,5-bisphosphate (PIP2). As a consequence of this cleavage, the signalling molecule $IP_3$ is formed which is the key molecule that regulates calcium release via $IP_3$ receptors (35,104,178,179). The formation of $IP_3$ at the plasma membrane of the cell results in the diffusion of $IP_3$ further into the cytosol until it reaches the membrane of intracellular $Ca^{2+}$ stores (180). $IP_3$ then binds to its receptor localised within the membrane of calcium stores such as the ER which results in a sudden release of $Ca^{2+}$, as a consequence, the concentration of $Ca^{2+}$ in the cytosol drastically increases.

As demonstrated in Fig. 1.5 this physiological release of $Ca^{2+}$ is carried out in repetitive transient spikes of $Ca^{2+}$ concentrations to produce cellular instructions (180,181). These oscillatory $Ca^{2+}$ signals not only serve as a signal for one function but can control several effects that are unique to the cell type. The $IP_3$ pathway has been shown to be utilised in numerous different cell types around the body (182–184). Specifically within PACs, the release of digestive enzymes is induced by the $IP_3$ pathway (34). Multiple different subtypes of $IP_3$ receptors have been found with different subtypes being expressed in certain cells. During knockout experiments in mice it was found that PACs are dependent on $IP_3$ signalling mediated by $IP_3R$ subtypes 2 and 3 (185).

### 1.12 NAADP Mediated Calcium Release in PACs

In comparison to ACh, CCK stimulates a different signal transduction pathway which utilises the messenger molecules NAADP and cADPR (35,143,176,177,179,186,187). In a similar fashion, CCK binds to its CCK1 receptor on located on the basolateral membrane of the cell (168,188). As a consequence, two secondary signalling messenger molecules are recruited, both cADPR and NAADP (176,186,187). Following the binding of CCK and the activation of its CCK1 receptor, the concentration of cADPR and NAADP increases, however, the exact mechanism of this is still unclear (176,186,187,189). The production of NAADP and cADPR both involve the enzyme CD38 which is a multifunctional, membrane bound enzyme (176,187). This is due to the complex nature of the enzyme which seems to be able to produce differing products depending on its location and pH during the reaction (176,189). As a consequence, it's unknown how the activation of CCK1 stimulates CD38 to produce cADPR and NAADP.

The resulting increase in cADPR and NAADP concentrations induces the opening of Ryanodine Receptors (RyR) present on the membrane of calcium stores, such as the ER (176,187,190–192). Although many studies have documented this correlation for several years, it's also unclear how these messenger molecules specifically

activate/open RyRs to release $Ca^{2+}$ especially as there is no logical binding sites for NAADP to RyRs (176,189). There have been several mechanisms suggested but none have been fully agreed as a consensus by the scientific community. The latest emerging mechanism which is suggested involves an intermediary protein which binds to the released NAADP which then activates RyRs (193). Whilst cADPR directly sensitises RyRs to release the stored calcium or perhaps sensitising the receptor enough to allow activation by NAADP, however, this is not known (176,187,189). Due to the complexity of calcium release within PACs, as multiple separate mechanisms affect each other, it is difficult to directly identify this unknown mechanism of RyR activation. Additionally, during these physiological signalling events, studies have documented the release of calcium from zymogen granules and lysosomes as well as the ER (176,189,193–195). Furthermore, it has been shown that this calcium release from zymogen granules and acidic stores can also be stimulated by $IP_3$ (193,196,197). Furthermore, experiments by Gerasimenko *et al.* (193) have highlighted the release of calcium from the nuclear envelope via $IP_3$, cADPR and NAADP stimulation.

Relatively recent studies have provided new evidence to contribute to the current hypothesis of NAADP induced calcium release. This evidence documents the release of $Ca^{2+}$ from endosomes/lysosomes when stimulated with NAADP, however, this release was mediated via Two-Pore Channels (TPCs) (35,177,189,194,195,197–200). Strong evidence has shown that NAADP mediated calcium release is heavily dependent on the expression of TPCs. The targeting of TPC family of proteins by NAADP has been well documented within recently carried out research, especially those investigating the role of $Ca^{2+}$ signalling during lysosomal defects and diseases (198,199,201–205).

NAADP was displayed to rely on both TPC type 1 channels as well as type 2 TPCs (198,202,204). Then again, recent research in PACs specifically demonstrated that NAADP induced calcium signalling is drastically reliant on the activation of TPC2 with subtle changes in calcium signalling when TPC1 was tested (35,177). On the other hand, the dependency on RyR subtypes 1 and 3 was much greater than that

of TPCs in order to elicit sufficient $Ca^{2+}$ release (35,177). Similar questions about the activation of TPCs are being asked as previously questioned relating to the activation of RyRs by NAADP. A potential theory for this is the reliance of $Ca^{2+}$-Induced $Ca^{2+}$ Release (CICR) which is another powerful pathway which cells utilise to amplify cytosolic calcium signals (35).

### 1.13 Intracellular Calcium-Induced Calcium Release

Once an agonist triggers an initial release of calcium into the cytosol, an amplification process starts to intensify the signal. The release of calcium extenuates the calcium signal by increasing the open state probability of surrounding $IP_3$Rs and RyRs therefore, increasing calcium release (35,206). This further release and action of calcium acts as a self-fortifying cycle that amplifies the calcium signal as shown in Fig. 1.6. However, this perpetual release of calcium comes to an end as $Ca^{2+}$ ions have dual effects on $IP_3$ and Ryanodine receptors as once calcium levels reach a certain concentration, it starts to become inhibitory and so closes these channels, preventing further release of calcium (35,206–208). This positive feedback mechanism of CICR is vital for all calcium signals within PACs and is stimulated regardless of whether ACh or CCK is the primary initiator (35,209). This co-operation between both $IP_3$ and NAADP signalling pathways allows cells to create these complex oscillatory spiking patterns. The amplificatory and inhibitory actions of $Ca^{2+}$ ions allow the sudden increase and peaking of calcium concentration which then facilitates the reabsorption of $Ca^{2+}$ back into stores via calcium pumps such as SERCA (35,125). As soon as channels releasing stored calcium close, pumps within the ER membrane are able to recapture the previously released calcium, therefore, decreasing cytosolic calcium levels as well as restoring stored reserves of $Ca^{2+}$ (35). This reduction in cytosolic calcium then permits the regeneration of a new signal and so continues the cyclic nature of CICR can continue.

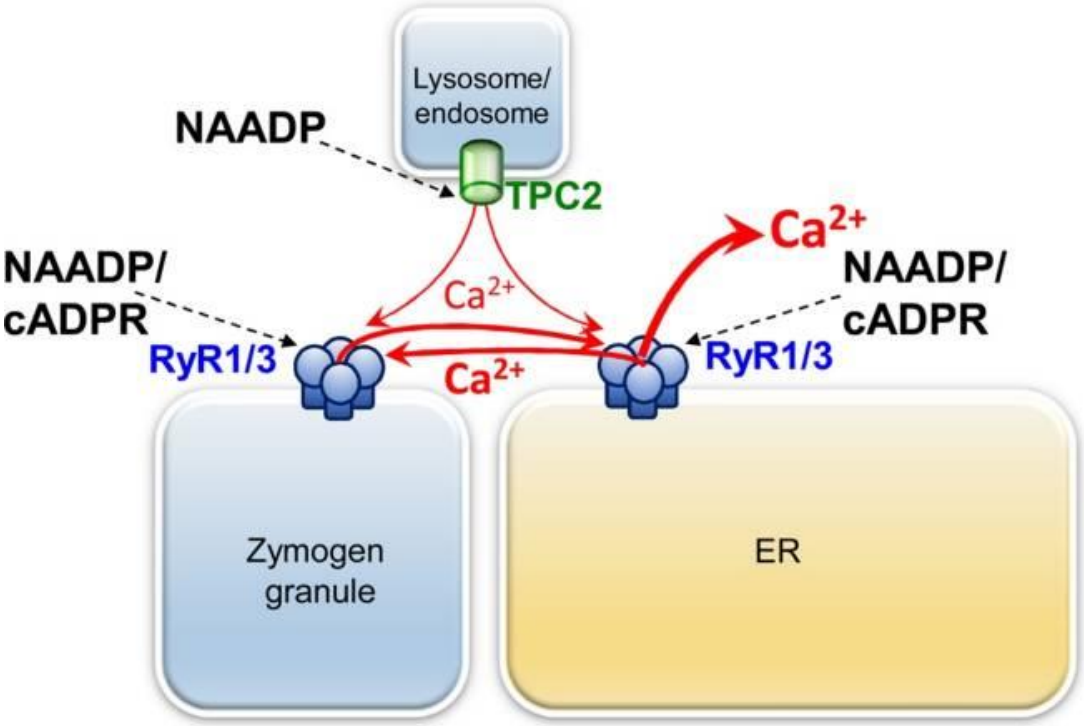



***Figure 1.6 Calcium Induced Release in PACs between intracellular stores induced by NAADP.***

*CICR is also initiated by the signalling molecule NAADP which has demonstrated activation of RyRs types 1 and 3 in both the ER and Zymogen Granules. Furthermore, this NAADP also triggered the release of $Ca^{2+}$ from acidic stores such as endosomes/lysosomes. As a consequence of this $Ca^{2+}$ release, local calcium ions induce further release of calcium from neighbouring RyRs in the ER and Zymogen Granules. Modified from Gerasimenko et al.* (177).

Furthermore, the initial stimulation of $IP_3$, cADPR or NAADP calcium release is located in the apical region of PACs due to the high concentration of calcium release channels in the apical pole (207). During the initial release of calcium and further release via CICR in the apical pole, a progressive wave of $Ca^{2+}$ moves towards the basolateral pole of the cell. However, during studies which involved the uncaging of local $Ca^{2+}$, local CICR was not produced in the basolateral pole, therefore presenting the requirement of both $IP_3$ and Ryanodine receptors (97,115,210). As the distribution of these receptors are different throughout the cell, CICR in the basolateral pole is not stimulated (112,113,210). These complex positive and negative $Ca^{2+}$ interactions between $IP_3$Rs and RyRs are essential for physiological calcium signal generation (35,71,123). The delicate balance of cytosolic calcium levels is invaluable and allows PACs to carry out their cellular functions. This is well documented during sustained stimulation when a consistent high concentration of intracellular calcium is portrayed (206). An elevated plateau of cytosolic calcium represents the balance between the influx of calcium into the cytosol and the extrusion of calcium back into stores and out the cell via SERCA and PMCA pumps (125,148,150,151,197,211,212). Upon the removal of the sustained stimulation, the cytosolic concentration of calcium is quickly restored to its resting level (111). Although the concentration of the cytosol is restored quickly following sustained stimulation, it takes a much longer time to enable the intracellular stores, such as the ER, to fully restore their internal concentration of $Ca^{2+}$ (213). This is on the contrary during physiological short spikes of cytosolic calcium where it's been shown that the concentration of $Ca^{2+}$ within the ER varies very little (110,214).

During sustained stimulation with ACh, plateau of elevated $[Ca^{2+}]i$ represents a delicate balance of $Ca^{2+}$ entry through store-operated $Ca^{2+}$ channels (SOCs) in the basal membrane (128,129)

### 1.14 Calcium Extrusion and Entry in Pancreatic Cells

The extrusion of calcium from the cytosol to extracellular stores via PMCA pumps has briefly been discussed, however, during calcium signals calcium ions also flow into the cytosol from extracellular stores during the amplification of calcium signals (159,160). The regulation of calcium entry and extrusion from the cell is another key part of the complex process of calcium signalling. Furthermore, calcium entry and extrusion are vital during physiological calcium signalling and pathophysiological signalling in PACs.

The plasma membrane of the cell is impermeable to $Ca^{2+}$ ions, therefore, $Ca^{2+}$ can only pass through the membrane via specific channels and receptors. This restriction allows the cell to regulate its calcium levels and to maintain homeostasis. The PMCA pumps present in the cell membrane are not only beneficial for maintaining the low resting concentration of calcium in the cytosol but can also re-establish intracellular $Ca^{2+}$ concentrations following a challenging rise of cytosolic $Ca^{2+}$ concentration within the cytosol (159,160). The majority of these PMCA pumps are localised towards the apical pole of the cell in order to extrude calcium during localised calcium spiking. However, during the restoration of normal cytosolic calcium levels, the intracellular stores are depleted of calcium (215). As a consequence, the extruded calcium is returned intracellularly to the ER from the extracellular fluid. Interestingly, this is primarily carried out within the basolateral region of the cell where the newly recuperated calcium ions are then returned to the ER (216). The group of channels that transport $Ca^{2+}$ ions from extracellular stores internally are known as Store-Operated Channels (SOCs) (94,159,160,163). As suggested by their name, SOCs dynamically respond to the state of intracellular stores. These channels are commonly activated by the release of $Ca^{2+}$ from intracellular stores which normally involves G-proteins and produces $IP_3$. These SOCs are commonly found within the basolateral membrane of the cell and often feed extracellular calcium directly into the ER (159,160,163). A well-known set of channels which functions as SOCs are $Ca^{2+}$ Release-Activated $Ca^{2+}$ (CRAC) channels. CRAC channels are activated by the depletion of free $Ca^{2+}$ within the ER as opposed

the concentration of cytosolic calcium or the concentration of total calcium within the ER (160,163,217,218).

CRAC channels are a complex structure, normally consisting of two main protein families (219). Commonly made up of Orai proteins and Stromal Interaction Molecule (STIM) proteins, CRAC channels have multiple physical characteristics including a low unitary conductance whilst also being extremely sensitive to $Ca^{2+}$ ions (159). These characteristics are due to the construction of the CRAC channel. The Orai1 molecule is the primary constituent of the ion channel pore section of the channel. This molecule and section are an integral part for CRAC channel activity and its selectivity for $Ca^{2+}$ ions (220,221). The STIM component of the channel commonly functions as the channel's ER calcium sensor. Furthermore, it acts as the activator of the CRAC channel. The identification of these constituent proteins of CRAC channels, Orai1 and STIM, shone light upon the molecular mechanisms involved during SOCE (220–226). Furthermore, following their identification, numerous studies were sought to understand the pore architecture of the CRAC channel and how it influences the influx of $Ca^{2+}$ into the cell.

The vital component of producing the CRAC current (ICRAC), Orai1, was first found during studies of T-lymphocytes (227). These studies were utilising positional cloning and linkage analysis from Severe Combined Immune Deficiency (SCID) syndrome patients (220,227). The Orai1 protein is made up of four transmembrane domains, aptly named TM1-TM4. Both transmembrane domain N- and C- termini are located within the cytosolic side of the channel. These termini regions also consist of STIM1 binding sites which are obviously vital for communication between calcium stores and CRAC channel sites as shown in Fig. 1.7 and Fig. 1.8. Multiple studies have reported the importance of Orai1 as an essential pore subunit of the channel (228). This was done by monitoring the effects of several mutations within the transmembrane domains which subsequently had profound effects upon the channel's ion selectivity and permeation (228–232). Upon further study, the structured assembly of the Orai channel is composed of an hexameric arrangement in concentric layers which enables the influx of calcium through a central ion pore

(233,234). Although we have learnt a great deal about this channel and the importance of the Orai1 subunit, the exact mechanisms which keep the pore closed in a resting state is still unknown (233,234).

The STIM1 protein is predominately located within the membrane of the ER with a luminal $NH_2$ terminus whilst having a cytoplasmic COOH terminus. The protein contains multiple different interaction domains within its 77kDa structure (222,225,235). The N-terminal region of the STIM1 protein within the ER lumen contains a sterile α-motif (SAM) in addition to an EF-hand (EF) calcium binding motif. This calcium binding motif functions as the protein's calcium sensor within the ER whilst the SAM is important during the regulation of STIM oligomerization which is a key step in the activation of the ORAI channel (222,225,236,237). On the opposing end of the protein, a region consisting of approximately 100 amino acids, is extremely important in the role of channel activation. The CRAC activation domain (CAD), also known as the STIM-Orai activation region (SOAR), is a polybasic domain at the very end of the C-terminus which directly interacts with the cellular plasma membrane at ER-Plasma Membrane functions (229,238–242). The binding of the polybasic domain and the Plasma Membrane activates the ICRAC (163,239). The distribution of STIM1 within the cell is also important to its functioning role as its location changes upon the depletion of calcium in stores. Prior to store depletion, the vast majority of STIM1 is located within the ER membrane whereas following store depletion, STIM1 translocates to distinct junctions between the ER and plasma membrane of the cell (221,222,225,235,237,243–247).

As alluded to earlier, this translocation of STIM1 is a key component of SOCE as the initial activation of SOCE is dependent upon the distribution of STIM and Orai within the cell. During the resting state of a cell and under physiological conditions, STIM1 and Orai1 are dispersed respectively throughout the ER and plasma membrane (160,222,225,237,239,248–252).

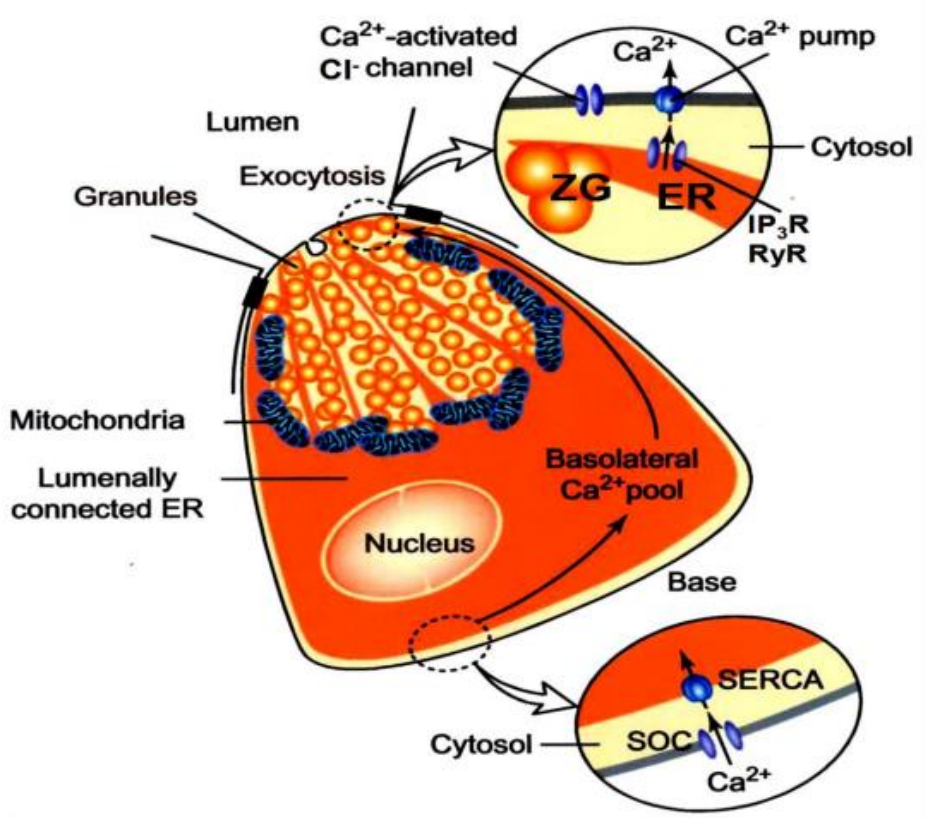



***Figure 1.7 Schematic diagram of organelle location within PACs***

*Highlighting the organisation of organelles in the cytosolic environment. The granular area located towards the lumen contains a high concentration of zymogen granules which are surrounded by a mitochondrial belt. Adapted from Petersen et al.* (110).

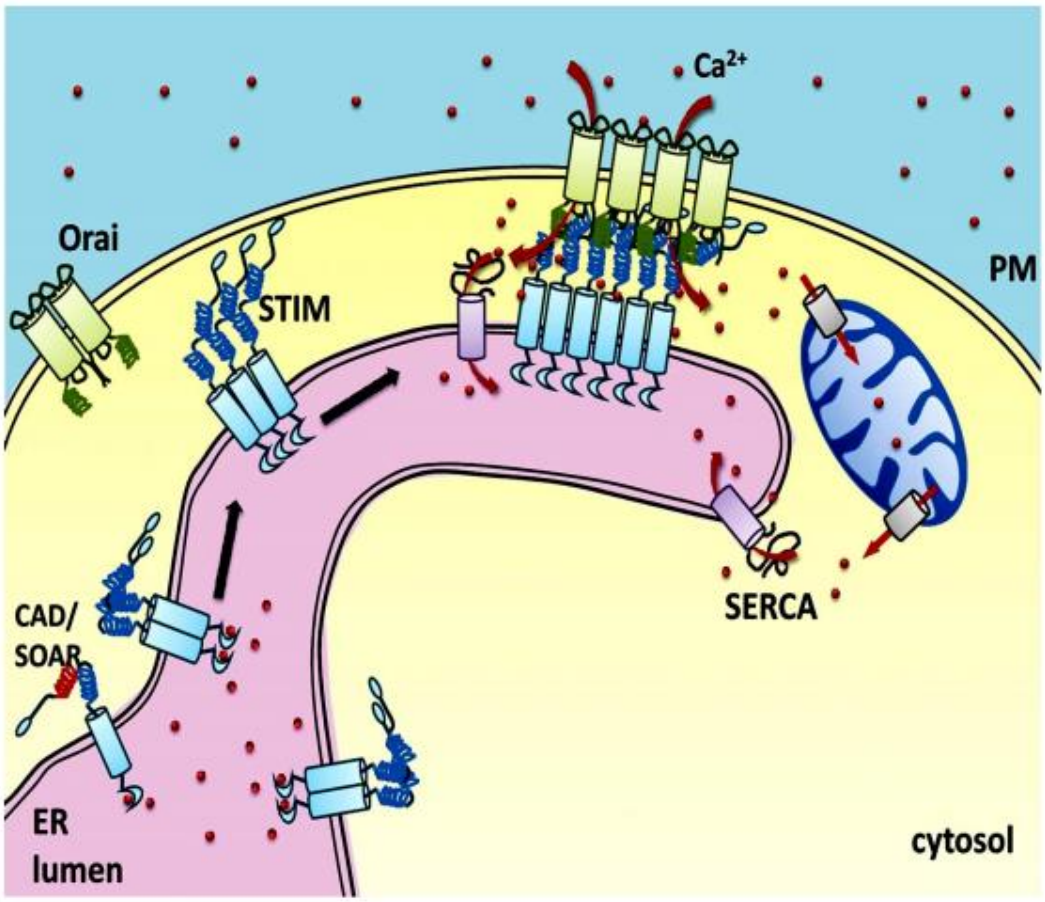



***Figure 1.8 Mechanistic view of Store-Operated $Ca^{2+}$ Entry involving STIM***

*Depiction of the molecular mechanisms involved during SOCE. During resting state, $Ca^{2+}$ is bound on the luminal side which maintains STIM inactivity. However, upon calcium depletion of the ER, $Ca^{2+}$ dissociated from STIM causes the translocation of STIM towards the ER-PM junction. At this point, the CAD/SOAR of STIM interacts with ORAI channels which in turn facilitates the entry of $Ca^{2+}$ into the cytosol and ER. Diagram modified from Shen et al. (242).*

However, when challenged by pathological stimuli, PACs can become overloaded with inappropriate $Ca^{2+}$ entry across the basal membrane. PMCA is unable to adequately deal with this influx due to its limited capacity (5). The half-maximal rate of calcium extrusion depends on $[Ca^{2+}]i$ of approximately 200 nM, but calcium extrusion mechanisms are almost completely saturated when $[Ca^{2+}]i$ is above 400 nM (253). PACs almost exclusively rely upon PMCA to extrude excess intracellular $Ca^{2+}$ from the cytosol in order to maintain and restore physiological calcium levels. However, extensive cytosolic $Ca^{2+}$ overload leads to disruption of this extrusion mechanism. Despite excessive calcium entry during pathology, it is well known that calcium entry is required for PACs' sustained secretory response and exocytosis and so requires the presence of external $Ca^{2+}$ (87,254).

### 1.15 Role of Calcium in Mitochondria Functioning

As already previously hinted, the mitochondrial not only plays a major role in supplying sufficient ATP for the cell but is also heavily involved in cellular calcium signalling. The vast cellular functions of the cell are dependent upon ATP, such as exocytosis. Although exocytosis relies on ATP, it is initiated by a rise in intracellular calcium (118,255). PACs rely heavily upon mitochondria functioning correctly due to the multiple functions that PACs demand of mitochondria. As mitochondria are needed to meet the cellular energy requirements and regulate $Ca^{2+}$ homeostasis, the organelle has evolved by incorporating the two functions together. Mitochondria have evolved the specific mechanism which affects energy production depending upon the change of intracellular calcium concentration. For this reason, mitochondria are located in three specific regions within the cell. These are the perinuclear, sub-plasmalemmal and peri-granular (116,166,256,257). As highlighted in Fig. 1.2 and Fig. 1.7, the distribution of mitochondria are primarily surrounding areas which are heavily involved in calcium signalling.

The process of exocytosis requires ATP to function but also requires calcium signals elicited by ACh or CCK. Furthermore, these calcium signals increase ATP consumption and production. These signals and the production of ATP can be monitored using several techniques. For example, the process of ATP production involves the oxidative phosphorylation of Nicotinamide Adenine Dinucleotide (NADH) which can be used due to its auto-fluorescence. As a consequence, NADH levels are a key indicator of live cell metabolism (258,259). Another part of the evolution of mitochondria is the concurrent repetitive rises of NADH concentration in line with cytosolic calcium oscillations (260). This, however, only occurs during physiological calcium oscillations and concentrations. If there is a sustained elevation of cytosolic calcium, a key stage in PAC pathology, then a single large transient of NADH is produced (260). Due to this, the process of small physiological spiking events of calcium, and so NADH, is a much more efficient way of producing sufficient ATP for cellular functions. Whereas sustained high intracellular calcium concentrations, which are indicative of pathology and are triggered by pathological

stimuli, are too large to enable mitochondria to efficiently produce suitable ATP levels to satisfy cellular energy requirement (260).

Despite the increased ATP consumption due to physiological signalling, cytosolic ATP levels actually increase. This is due to the ATP levels dependence upon oxidative phosphorylation in the mitochondria (261). As a consequence of this, when this oxidative phosphorylation is inhibited, physiological cytosolic calcium signalling induced by ACh or CCK causes a reduction of cellular ATP (261). The uptake of $Ca^{2+}$ into the mitochondria via the Mitochondrial Calcium Uniporter (MCU) during these calcium signalling events is preceded by a rise in local $Ca^{2+}$ in the apical pole which is very quickly followed by a rise in mitochondrial $Ca^{2+}$ (262–264). Subsequently, three dehydrogenase enzymes, pyruvate dehydrogenase, $NAD^{+}$-isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase, are stimulated by the increased calcium levels. These enzymes are all involved in the ATP producing mechanism, the Krebs cycle (265,266). The influx of calcium into the mitochondria is a relatively fast process compared to its efflux. The movement of calcium out of the mitochondria is a slow process and so mitochondrial ATP production remains high due to its constant stimulation by the increased $Ca^{2+}$ concentrations. This is an important mechanism as this increased ATP production is not only required to produce sufficient energy for secretion but also to supply the $Ca^{2+}$ extrusion pathways via PMCA in addition to $Ca^{2+}$ uptake into the ER via SERCA (116,215).

Additionally, the transfer of calcium ions from the ER into mitochondria is facilitated by junctions linking the two organelles as shown in Fig. 1.9. The $IP_3$Rs and VDACs are closely associated within these junctions. Furthermore, there are specific proteins which bind the two organelles closely together such as mitofusins (MFN) which bind the two membranes whilst the chaperone protein, glucose-regulated protein 75 (grp75), couples the ER localised $IP_3$R to the mitochondria localised VDAC (255). This coupling aids the transfer of calcium ions from the ER to the mitochondrial matrix.

As previously mentioned, the distribution of mitochondria are relative to the cellular locations which often require ATP as shown in Fig. 1.2 and Fig. 1.7. Distinct groups of mitochondria surround these ATP requiring areas. The apical granular located mitochondria are vital in localising cytosolic calcium elevations within the apical area, therefore, these mitochondria act as a $Ca^{2+}$ buffer barrier. Furthermore, as previously mentioned, this $Ca^{2+}$ buffering also induces elevated local ATP levels. The increased ATP requirement for $Ca^{2+}$ extrusion is also fulfilled by mitochondria primarily located in the apical area. Conversely, the peripheral group of mitochondria are important in supplying both SOCE at the base of the cell and $Ca^{2+}$ pumping into the ER. Finally, a third group of mitochondria surround the nucleus of the cell which confines localised calcium signals within the nucleus but also protects the nucleus from unwanted calcium signals from neighbouring areas (116).

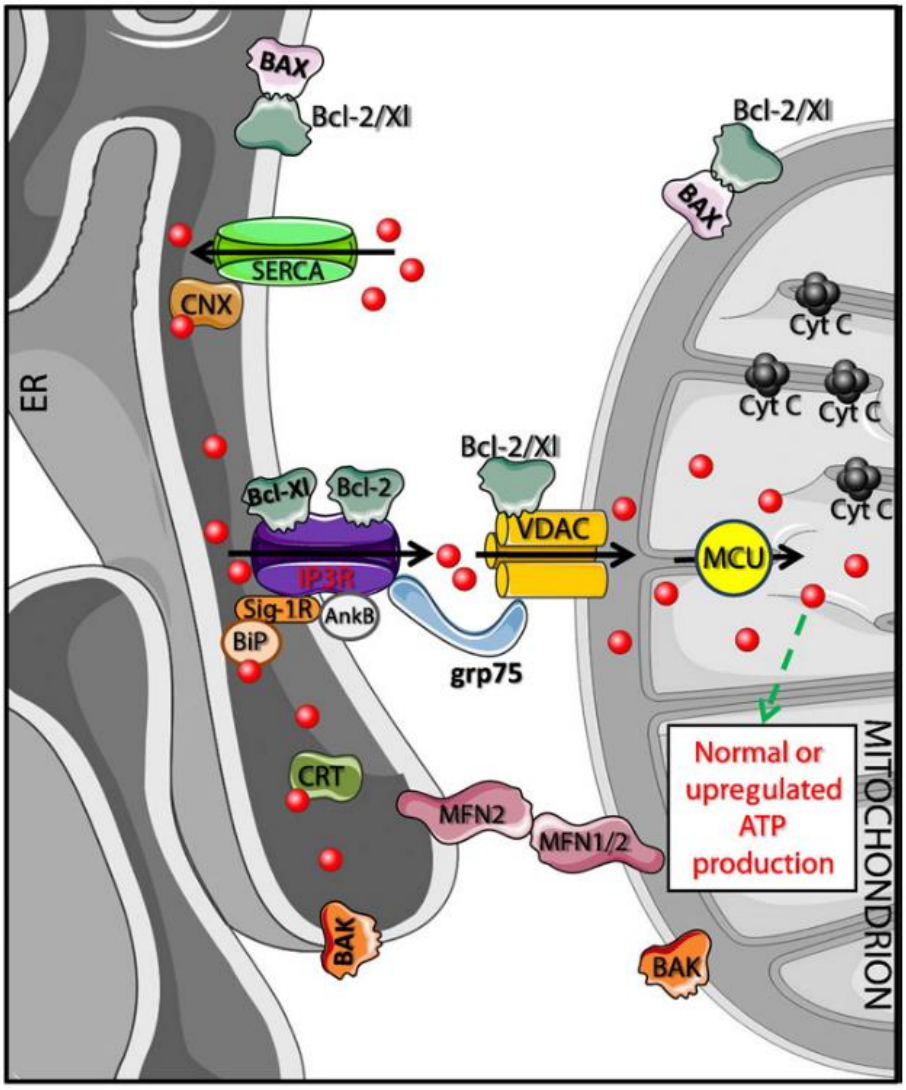



***Figure 1.9 Schematic representation highlighting the structural coupling between the ER and mitochondria.***

*The transfer of $Ca^{2+}$ ions (red dots) between the ER and mitochondria is a vital process and part of physiological calcium signalling in order to aid ATP production within the mitochondria. This transfer of ions occurs at junctions between the two organelles which are facilitated by proteins such as mitofusins and grp75. These proteins bind the membranes of the two organelles together which supports the transfer of ions from the ER to the mitochondrial matrix. Diagram modified from Decuypere et al. (255).*

### 1.16 Calcium Signalling in Pancreatic Stellate Cells

In the 1980s the myofibroblast-like described cells which were woven in between clusters of PACs were deemed by Watari *et al.* (267) to be Pancreatic Stellate Cells (PSCs) as shown in Fig. 1.10. These cells make up approximately 4% of the cells within the pancreas (268). The normal physiological function of these cells is still unknown and were initially vastly regarded as being quiescent. The majority of the very little work carried out upon PSCs has been based upon their quiescence and has focused on the activation of PSCs. This activation of PSCs has commonly been associated with pathological scenarios such as pancreatic cancer and chronic pancreatitis (269–271). This association will be discussed later. During physiological signalling, very little is known about PSCs. Previous work studying cultured PSCs demonstrated substantial cytosolic calcium signals in response to high concentrations of Bradykinin (BK) among some other substances (36). However, the substantial work carried out by Gryshchenko *et al.* (272) investigated this generation of intracellular calcium signals whilst being able to make extensive comparisons between PSCs and PACs. This provided some intriguing results. The study of PSCs within a physiological microenvironment enabled the measurement of calcium responses to low doses (100 pM) of BK whilst cultured cells required much greater concentrations in order to yield a response. This is extremely interesting with implications in relation to the activation of PSCs. The threshold for activating normal PSCs is similar to the normal levels of plasma levels of BK in the range of 40-70 pM (273,274). Therefore, any conditions which produce BK levels higher than that of the normal range would induce intracellular calcium signals within normal PSCs.

The work conducted by Gryshchenko *et al.* (272) revealed the activation of bradykinin type 2 (B2) receptors during BK stimulation in addition to the reliance upon calcium entry during stimulation. The initial stimulation of pancreatic tissue with BK produces a distinct signal within PSCs whilst simultaneously evoking no response in PACs. The initial BK induced signal triggers the release of $Ca^{2+}$ from internal stores which can be further blocked by the inhibition of phospholipase C or

$IP_3$ receptors. Following the primary release of $Ca^{2+}$ the second phase of the calcium signal involves the opening of CRAC channels which allow the influx of $Ca^{2+}$ (159,275). Gryshchenko *et al.* (272) demonstrated that the use of the CRAC channel inhibitor, GSK-7975A, successfully prevents this secondary phase of calcium influx during BK-induced signalling in PSCs. Furthermore, Gryshchenko *et al.* (272) highlights that the blockage of B2 receptors not only prevents BK-related signalling but also shows reduced cell death in PACs, despite PACs' inability to respond directly to BK stimulation. Therefore, suggesting that this protective effect may be due to the inhibition of $Ca^{2+}$ signal generation within PSCs.

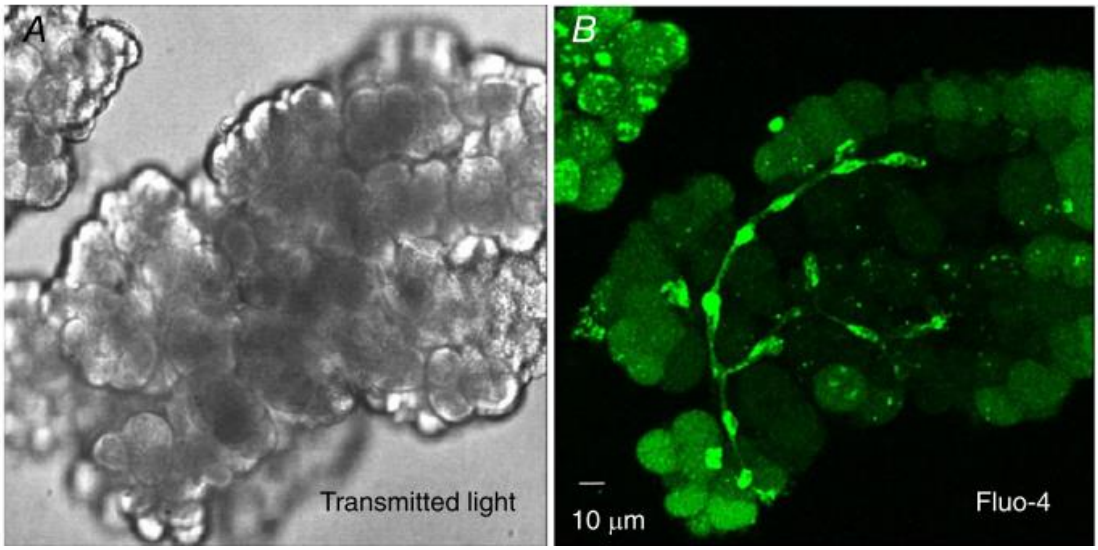



***Figure 1.10 Images of pancreatic lobules highlighting pancreatic stellate cells.*** ***(A)*** *Transmitted light image of an isolated pancreatic lobule similar to those used in this study when investigating PSCs.* ***(B)*** *Fluorescent image of the same pancreatic lobule in (A) which was loaded with Fluo-4. When stimulated with a specific wavelength of 488nm, PSCs become much more distinct compared to the PACs in the surrounding lobule. Images presented from Gryshchenko et al. (272).*

### 1.17 Pancreatic Acinar Cells in Acute Pancreatitis

As previously alluded to, PACs play a major role during the development of Acute Pancreatitis. This well-known during multiple different triggering factors of Acute Pancreatitis shown in Table 1.1. The two most common causes of Acute Pancreatitis are either alcohol-related or gallstone-related (5). The introduction of bile acids into the pancreatic duct due to the blockage of biliary ducts from the gallstone has shown pathological effects upon PACs (5,19,20,34,131,276,277). The excessive concentrations of $Ca^{2+}$ in the cytosol overloads several key processes within the cell which are necessary for its survival (34). This cytosolic overload of calcium also overloads the mitochondria which drastically reduces ATP production whilst the high concentrations of calcium ions also prematurely activates proteases within the cell (4,276,278). This destroys the internal cellular mechanisms and in doing so, induces unwanted cell death and necrosis. This process then releases the cell contents to local environment which stimulates local inflammation. As consequence, this induces the development and worsening of acute pancreatitis (35). During the induction of any calcium overload involves similar calcium signalling pathways and mechanisms, however, some specific mechanisms/receptor targets are uniquely involved depending on the initial trigger which will be discussed.

### 1.18 Pancreatic Stellate Cells in Acute Pancreatitis

PSCs have recently been shown to play a potentially substantial role in pathological fibrosis of the pancreas (172,279). Studies have demonstrated that the production and secretion of extracellular matrix proteins greatly increased in response to alcohol and bile acids. Additionally, PSCs can change between states from a quiescent phenotype to an activated phenotype similar to hepatic stellate cells (280). When in an activated state PSCs express α-SMA and produce large quantities of extracellular matrix proteins including collagen type I and III, laminin and fibronectin (269). The current consensus of the scientific community is that the activation of PSCs is not directly involved in the initial development of acute pancreatitis. Rather its progressive development as the increased production of extracellular matrix increases the fibrosis within the pancreas therefore worsening the condition of the pancreas (268). The specific mechanism of PSC activation is unclear; however, recent studies have demonstrated that excessive bile acid and alcohol can induce extreme intracellular $Ca^{2+}$ concentrations. Consequently, these excessive $Ca^{2+}$ signals lead to the damaging effects of acute pancreatitis as highlighted in Fig. 1.11 (172,279).

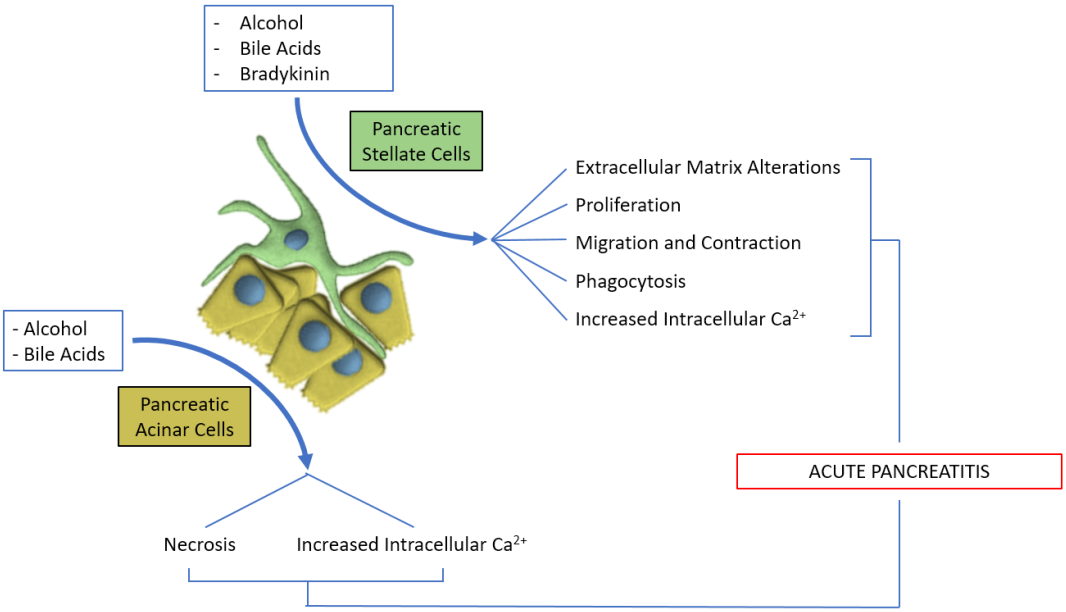



***Figure 1.11 Schematic diagram highlighting various pathways in which acute pancreatitis is induced via PACs and PSCs.***

*The effects on pancreatic acinar and stellate cells are clearly different with pathogenic stimulations on PACs mainly inducing necrosis or internal calcium overload. Whereas pathogenic stimulations in PSCs primarily induces activation, the production of extracellular matrix proteins and proliferation. These effects are all involved in the development of acute pancreatitis, specifically, pancreatic fibrogenesis.*

Furthermore, it is theorised that PSCs may also play a role in the deterioration of acute pancreatitis and in the development of chronic pancreatitis (38). Primarily, this theory is based on there being a difference in signalling between pancreatic acinar and stellate cells. The inflammatory mediator peptide BK has demonstrated stimulation of calcium signalling specifically within PSCs whilst having no effect upon PACs (38). Bradykinin elicited an increased intracellular $Ca^{2+}$ concentration which activated PSCs which also induced proliferation and secretion of extracellular matrix proteins. It was initially speculated that this BK-elicited activation of PSCs may indirectly induce signalling within neighbouring PACs. However, the study by Gryshchenko *et al.* (38) showed that the BK induced $Ca^{2+}$ signals within PSCs did not elicit any calcium signalling within nearby PACs. Nevertheless, it has been indicated that other types of signal generation may be involved (272). This study provided evidence suggesting that the inhibition of Bradykinin B2 receptors greatly reduced the extent of necrosis within PACs when evoked by pancreatitis-inducing agents not utilising the B2 receptor pathway.

Consequently, this finding potentially indicates that Bradykinin or the Bradykinin B2 receptor plays a role in the development of acute pancreatitis via specific mechanisms on PSCs which are not yet fully understood. However, important work carried out by Ferdek *et al.* (172) demonstrated the effects of bile acids and other pathological agents upon PSCs. Powerful intracellular calcium signals were recorded within PSCs during the application of bile acid salts, NaChol and TC which all additionally induced necrotic cell death. Despite this necrosis, surprisingly the neighbouring PACs were not deemed to be affected by the local cell death. Furthermore, these pathological calcium signals within PSCs were heavily reliant upon the presence of extracellular calcium which is in stark contrast to PACs. Additionally, it was found that PACs within their naturally occurring environment of lobules proved to be more resistant to pathological stimuli (172).

### 1.19 Alcohol Induced Pathology

The pathology of pancreatic cells during acute pancreatitis has been greatly studied. However, this pathology is initiated by various different pathological stimuli. Excessive alcohol intake has been a major cause of acute pancreatitis which many studies highlighting its correlation (5,281–284). The intake of more than 4 alcoholic drinks per day results in a greater than doubling risk of developing pancreatitis (285). In contrast, during experiments inducing acute pancreatitis, alcohol alone was not sufficient to induce acute pancreatitis. Only the application of CCK and alcohol induced acute pancreatitis in rats, however, it is thought that alcohol consumption sensitises the pancreas to other inducers of acute pancreatitis (286–288).

Additionally, a theory which builds on the process of alcohol-induced pathology is the inclusion of metabolic systems within the pancreas. The expression of Fatty Acid Ethyl Ester (FAEE) synthases in PACs synthesis FAEE from ethanol and free fatty acids which is the non-oxidative metabolism of alcohol within the pancreas (289). There is some evidence which indicates that high levels of acetaldehyde may damage the pancreatic tissue. Coincidently, alcohol dehydrogenase produces acetaldehyde during the oxidative metabolism of alcohol (290,291). Specifically, carboxyl ester lipase is a FAEE synthase that is also expressed in PACs and is one of the enzymes that is stored within zymogen granules in the apical region (292). In some studies, FAEEs were discovered in high concentrations within the liver and pancreas following alcohol abuse due to the high levels of FAEE synthases present (281). Although, the oxidative metabolite (acetaldehyde) has not shown pathology-inducing behaviour, the contrary is said regarding non-oxidative alcohol metabolites FAEEs. FAEEs have been significantly involved in inducing sustained global cytosolic $Ca^{2+}$ concentrations which leads to premature activation of digestive proenzymes within the cell (282). Furthermore, the application of FAEEs to PACs stimulated significant cellular necrosis, however, the application of similar concentrations of aldehyde did not yield similar findings (282,293).

During *in vivo* experiments, FAEEs have demonstrated toxicity as replicating the findings shown in *in vitro* studies results in increased trypsinogen activation, pancreatic oedema and cytoplasm vacuolisation, therefore suggesting the presence of these fatty metabolites (294). The reliance of the non-oxidative metabolite of ethanol and fats has clearly been shown to exert these toxic effects upon PACs compared to the sole application of ethanol or it's oxidative metabolite, aldehyde (282). A form of FAEE, Palmitoleic Acid (POA) Ethyl Ester (POAEE), was applied to PACs during multiple studies. The resulting findings indicated a large sustained elevation of cytosolic $Ca^{2+}$ concentration was caused due to the appliance of POAEE (295). The activation of $IP_3Rs$ and calcium release from the ER was documented during this cytosolic increase (295). Following which, the activation of SOCE mechanisms were also documented which further sustained the cytosolic calcium overload (282). However, a positive insight to this is the inhibition of these mechanisms. The use of the CRAC channel inhibitor GSK7975A produced a decreased cytosolic calcium overload in addition to reduced cell death (296,297).

The application of solely POA also resulted in a significant increase in cytosolic $Ca^{2+}$ concentration and in doing so, also induces cell death (282). On the other hand, the increase shown due to POA was much slower than the responses documented during the application of POAEE (282). Furthermore, the responses elicited by POA were not inhibited by caffeine nor were they changed upon the removal of extracellular calcium, whereas both these changes reduced calcium signalling induced by POAEE (282,295). This difference suggests that POA related calcium signalling is not primarily driven by $IP_3$ mediated calcium release or SOCE.

It's thought that the effects induced by POA and POAEE are due to the uncoupling effects on oxidative phosphorylation which impacts upon the mitochondria's capability to produce ATP. This disruption mainly impairs SERCA and PMCA function and so results in the failure to supply the cell's ATP demand and so induces cell death (295,298). The SERCA pump requires ATP to function and to move $Ca^{2+}$ ions back into the ER, additionally, PMCA requires ATP to pump $Ca^{2+}$ through the plasma membrane of the cell to counteract the influx of calcium from SOCE (295,298).

Furthermore, the malfunction of these two pumps, the vacuolar $H^+$-ATPase is unable to maintain the high concentration of calcium ions within acidic stores of the cell. As a consequence, the calcium within these stores also makes its way into the cytosol (283). Besides the effects of POA and POAEE upon mitochondrial ATP production, these agents are also able to depolarise the mitochondrial membrane (299).

### 1.20 Bile Induced Pathology

Initially the connection between Biliary diseases such as gallstones and acute pancreatitis has been contested for numerous years (300–303). However, currently it is thought that the overall mechanisms involved are well understood, yet there is still no viable treatment for bile-induced acute pancreatitis. The excessive cytosolic calcium overload is a key factor in the premature activation of trypsinogen and the induction of acute pancreatitis (278,304). Taurolithocholic Acid 3-Sulfate (TLC-S) is a component of bile acid which has been part of a multitude of studies in the past few decades during the investigation of bile-induced acute pancreatitis. Commonly, TLC-S has been applied in the micromolar range during experiments utilising pancreatic cells in order to initiate intracellular calcium signalling events (305). However, this level of TLC-S seems rather contradictory at a glance as investigations in patients with Chronic Pancreatitis demonstrated blood plasma levels of bile acids in the micromolar range compared to the millimolar range of bile acids found in the duodenum (306). Nevertheless, due to the design of experiments such as those previously carried out and those within this study, high concentrations of TLC-S are required in order to replicate pathological developments and signals within cells compared to relatively low bile acid concentrations found in blood plasma of diseased individuals (306). Therefore, the use of relatively high concentrations of TLC-S aims to replicate long term pathological developments found in patients within a short time frame in isolated pancreatic cells (305).

TLC-S has demonstrated an ability to initiate intracellular calcium signals within cells at low concentrations in addition to causing sustained calcium signals during the application of high concentrations (307,308). The triggering of calcium release from intracellular stores upon TLC-S application has not only been shown in PACs, but also in other cell types such as hepatocytes (277).

PACs are able to respond to the presence of TLC-S due to the occurrence of a TLC-S transport mechanism found within the plasma membrane of PACs (131). TLC-S is able to stimulate several different variants of calcium signals within PACs depending on the strength of stimulus. Local apical calcium spikes in addition to long-lasting calcium elevations and global transient oscillations are produced upon TLC-S application (131). When applied at low concentrations, TLC-S consistently demonstrated the generation of small oscillatory patterns whilst during the application of high concentrations, long prolonged calcium plateaus were much more evident (131,307–309). Whilst investigating this, it was suggested that these calcium signals induced by TLC-S involved the complex secondary messenger based mechanisms such as $IP_3$, cADP-Ribose and NAADP (99,131,193,209,310,311). However, this link was confirmed later by Gerasimenko *et al.* (309). Here, TLC-S exhibited the triggering of $Ca^{2+}$ release from intracellular acidic stores and the ER (131,309). Furthermore, these evoked signals were mediated via both RyRs specifically involving NAADP and $IP_3$Rs. However, cADPR was not deemed to be involved (5,309). Additionally, CICR was found to play a major role in the creation and amplification of elevated cytosolic calcium plateaus which also involved the influx of calcium as well as promoting the activation of trypsinogen within the cell (278,304,309). During these studies although the mitochondria was not shown to release calcium during the triggering of calcium signals, it has been demonstrated that the overload of calcium induced by TLC-S depolarises the inner mitochondrial membrane and so, reducing intracellular ATP levels which has a drastic effect upon the cell (131,295,309).

### 1.21 Asparaginase Induced Acute Pancreatitis

For some time, significant research has been undertaken into the main causes of acute pancreatitis, alcohol- and bile-induced. However, since the use of asparaginase in the treatment of Acute Lymphoblastic Leukaemia (ALL), some patients must cease ALL treatment due to the occurrence of acute pancreatitis. Acute Lymphoblastic Leukaemia is an aggressive cancer which progresses rapidly. Although ALL is an extremely rare condition and is unlike other types of leukaemia, it is the most common type of childhood leukaemia (312). Acute Lymphoblastic Leukaemia specifically targets white blood cells with the release and production of premature white blood cells known as blast cells. This increased release of blast cells also reduces the volume of red blood cells and platelets which leads to anaemia. Additionally, these blast cells have a reduced effectiveness in combating bacteria and viruses, therefore, increasing the vulnerability of patients to infections (313).

The enzyme asparaginase is a well-established drug for lymphoproliferative diseases in children over the past 40 years (314). The enzyme is normally paired with chemotherapy due to its non-association with any notable haematotoxicity (314). The specific mechanism of action of asparaginase is to catalyse the degradation asparagine into ammonia and aspartic acid. Asparagine is an essential amino acid and is critical for the cellular life cycle during DNA, RNA and protein synthesis. However, generally human cells are naturally deficient in endogenous asparagine. Therefore, this deficiency is normally offset with the use of the enzyme, asparagine synthase which produces asparagine from aspartic and glutamine acids (315). Lymphocyte cells are naturally deficient in asparagine synthase which prevents the lymphocytes producing asparagine endogenously. Therefore, lymphocytes are reliant on extracellular tissues and plasma as sources of asparagine (316). As a result, asparaginase is a very specific drug as it targets cancerous lymphoblastic cells as non-cancerous cells are able to produce asparagine endogenously. Therefore, inducing apoptosis in lymphocytes as the levels of endogenous asparagine are reduced further and so limiting the number of proliferating lymphocytes (315).

Asparaginase is used in all treatment procedures for ALL in children with an extremely high survival rate of nearly 90% in childhood (317). This is very much due to the effectiveness and specificity of the treatment (313). However, approximately 5-10% of ALL patients treated with asparaginase also develop acute pancreatitis which requires ALL treatment to cease until the acute pancreatitis has been treated.

Overall, the mortality rate of patients with acute pancreatitis is 5% but different mortality rates are shown amongst the different states of acute pancreatitis (318). Pancreatitis cases with necrotic tissue have a much higher mortality rate of 17% compared to cases with little necrosis at 3% mortality (13). Additionally, mortality rates are even greater in cases of necrotic pancreatitis which is infected compared to sterile necrotic pancreatitis (30% mortality vs 12% respectively). Consequently, the possible detrimental effects of acute pancreatitis are life threatening requiring the termination of asparaginase treatment of ALL. As a result, the treatment of the initial disease is significantly reduced (34).

### 1.22 Asparaginase-Induced Pathology in PACs

Until recently the induction of acute pancreatitis during asparaginase treatment was unclear and lacked investigation. However, the study by Peng *et al*. (34) demonstrated intracellular effects induced by asparaginase within PACs. The results shown are likely to be the causative mechanisms which lead to acute pancreatitis.

The study demonstrated that asparaginase stimulates elevated $Ca^{2+}$ concentrations within the cell which activates digestive enzymes whilst simultaneously decreasing cellular ATP. Consequently, both of these effects lead to cellular necrosis and inflammation of local tissue. Asparaginase transduces these elevated $Ca^{2+}$ concentrations through the Protease Activated Receptor 2 (PAR2) accompanied by signal transductions via $IP_3$ and NAADP signalling as shown in Fig. 1.12. As a result, asparaginase-Induced acute pancreatitis does not rely upon the presence of asparagine nor does it affect it. Consequently, this highlights several pathways which are involved during asparaginase-induced acute pancreatitis which may

provide an opportunity for therapeutic intervention whilst not affecting the treatment mechanism for ALL. Therefore, potentially preventing the development of asparaginase-induced acute pancreatitis during asparaginase treatment (34).

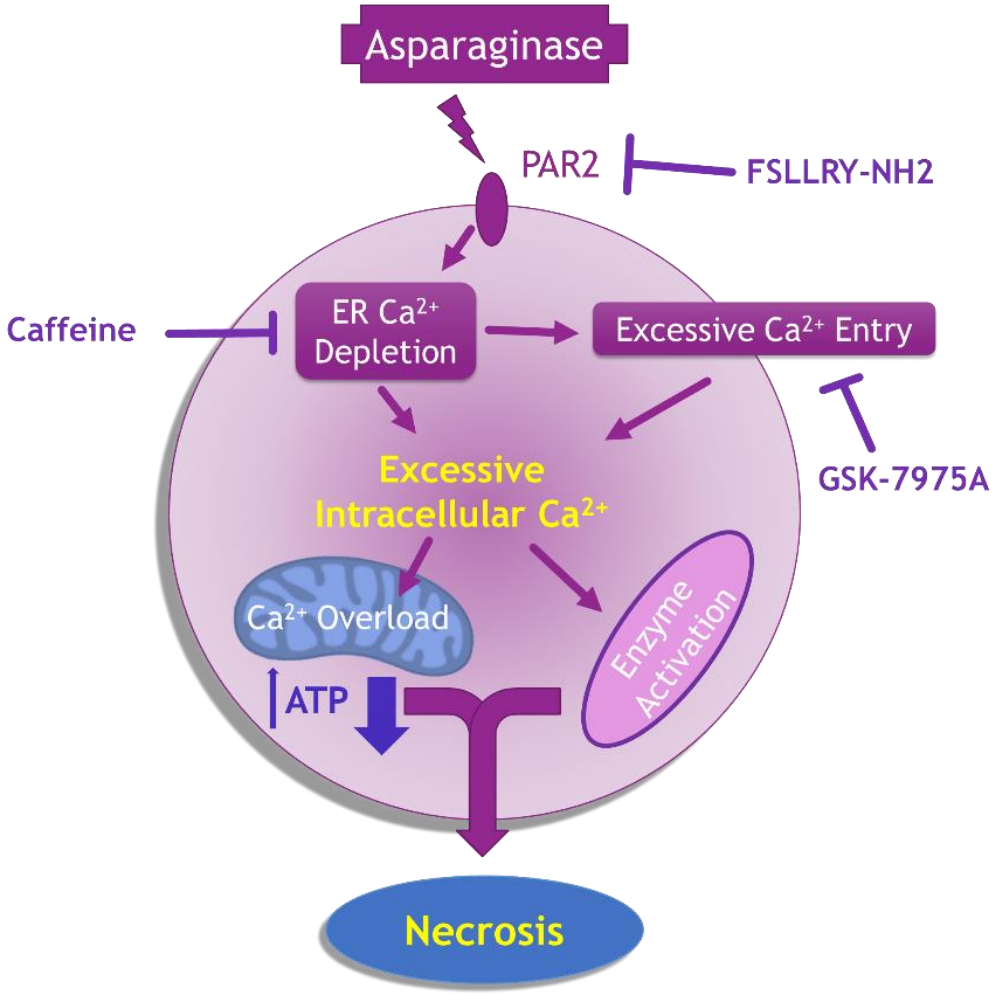



***Figure 1.12 Overview of asparaginase induced pathology within PACs.***

*An adapted cell model by Peng et al. (34) highlighting the processes involved in asparaginase induced pathology within PACs. Asparaginase is shown to activate the PAR2 receptor which in turn leads to excessive intracellular $Ca^{2+}$ release which in turn activates digestive enzymes and reduces cellular ATP, therefore, leading to necrosis and the development of Acute Pancreatitis. The model also demonstrates sites where this pathology can be inhibited such as the use of caffeine, FSLLRY-NH2 and GSK-7975A as inhibitory compounds.*

### 1.23 Role of PSCs in Asparaginase Induced Acute Pancreatitis

The very close relationship between pancreatic stellate and acinar cells is evident in the development of acute pancreatitis. Considering the studies previously mentioned such as that made by Gryskchenko *et al.* (272), the pancreatitis inducing effect of BK upon the pancreas clearly demonstrates a close relationship between PSCs and PACs during the development of acute pancreatitis. Despite this, the specific mechanisms underlying crosstalk between PACs and PSCs is still poorly investigated and understood. Further evidence of a complex relationship between pancreatic acinar and stellate cells has been the shown suppression of pancreatic cellular changes during pancreatitis due to the inhibition of B2 receptors within PSCs (38,273,319,320). With PACs being irresponsive to BK signalling, this evidence demonstrates that there must be communication between pancreatic acinar and stellate cells which may affect the development of acute pancreatitis. Peng *et al.* (34) highlighted the profound intracellular effects of asparaginase in PACs yet, there is no published study testing the effects of asparaginase in PSCs. Therefore, it does not seem unreasonable to speculate that PSCs may also play a role in the development of asparaginase-induced acute pancreatitis since there is a profound effect of asparaginase demonstrated by Peng *et al.* (34) within PACs. Investigation into whether PSCs are affected by asparaginase had not previously been done before preliminary experiments were completed during the last few years. The unpublished data from these experiments demonstrate that there was an overall sustained calcium response in PSCs to asparaginase shown in Chapter 4. However, the results did not highlight a clear pathway indicating how PSCs are involved with asparaginase-induced acute pancreatitis.

### 1.24 BH4-Bcl-2 Peptide as a Potential Therapeutic Tool

The anti-apoptotic B-cell lymphoma Bcl-2-family members are critically involved in maintaining mitochondrial integrity. This is commonly done by scavenging and inhibiting Bax and Bak1 which are pro-apoptotic Bcl-2-family members (321). This process involves the formation of a hydrophobic cleft by the Bcl-2 Homology (BH)

domains 1, 2 and 3 of the anti-apoptotic Bcl-2 proteins which interacts with the BH3 domain of the proapoptotic family members. Furthermore, the BH domain located closest to the N-terminal has also been shown to play a critical role in the anti-apoptotic properties that Bcl-2 presents (322–324). In recent years, the Bcl2 family of peptides have transpired to be important modulators of $IP_3$ and Ryanodine receptors (148,325,326).

It has been demonstrated that the regulation of intracellular $Ca^{2+}$ release channels are affected by the N-terminus of the BH4 domain. Specifically, Bcl-2 is expressed within the membranes of multiple organelles in the cell such as the mitochondria, nuclear envelope, vesicles and the smooth and rough endoplasmic reticulum (327,328). As a consequence of the locations of Bcl-2, $IP_3$Rs which located within the membrane of the ER are directly inhibited by the local Bcl-2 within the ER membrane (323,324). A protein complex is formed between the Bcl-2 and $IP_3$R specifically at the point of the central modulatory domain of the receptor. This complex then inhibits and prevents $IP_3$-mediated $Ca^{2+}$ release from the ER. Coincidently, this inhibition of calcium release may prevent the triggering of apoptotic cell death by preventing excessive calcium release. Although this inhibition is carried out by the Bcl-2 protein, only the specific BH4 domain of the Bcl-2 protein is required to sufficiently inhibit $IP_3$R-mediated $Ca^{2+}$ release. This inhibition of calcium release has shown great importance in preventing Mitochondrial Outer Membrane Permeabilisation (MOMP) which leads to cellular apoptosis (323,324).

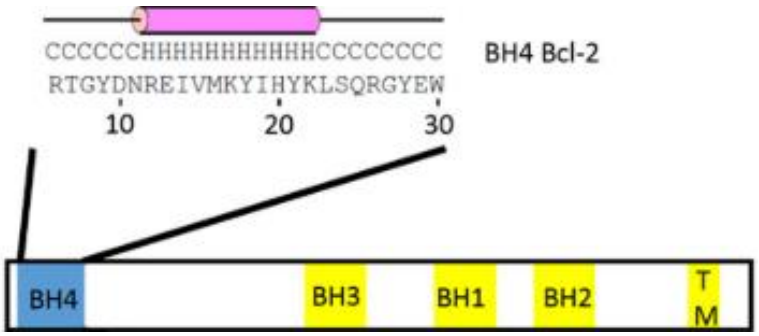



***Figure 1.13 Structural amino acid sequence of the BH4-Bcl-2 novel peptide.*** *Structural diagram of the Bcl-2 family of peptides and highlighting the different binding domains within its amino acid structure, including the domain of interest in this study, the BH4 domain. Modified from Vervliet et al.* (329).

In addition to BH4's potential to inhibit $IP_3$Rs, Vervliet *et al.* (329) and his group have demonstrated Bcl-2 proteins bind to and cause the inhibition of RyRs (326,330). This newly found interaction is again mediated by the BH4 domain of the Bcl-2 protein. Upon binding of the BH4 domain and the RyR, NAADP-mediated calcium signalling is inhibited (326,330). Following this, *Vervliet et al.* (329) sought to test whether or not these inhibitory effects of the BH4-Bcl-2 protein could reduce or even produce protective effects during cellular pathology (326,330). This thinking led to the experiments carried out in (326,329,330). This study demonstrated and confirmed multiple theories regarding the protective effects of the BH4 domain of the Bcl-2 peptide shown in Fig. 1.13. *Vervliet et al.* (329) decided to study the effects of the BH4-Bcl-2 peptide upon PACs due to the extensive research that has been produced regarding PACs' physiological and pathophysiology in relation to calcium signalling (96,305,309,331,332).

The study by Vervliet et al. (329) highlighted and confirmed several protective properties of the BH4-Bcl-2 peptide. During physiological CCK-mediated calcium signalling, cytosolic calcium levels were inhibited by the application of the BH4-Bcl-2 peptide by inhibiting RyR activity. Although this inhibition confirmed RyR inhibition, experiments failed to demonstrate inhibition of $IP_3$-mediated calcium signalling in PACs which could have been caused by multiple factors such as the potential degradation of BH4 domain into smaller fragments which were not able to inhibit $IP_3$Rs but may have been sufficient to disrupt Ryanodine-mediated signalling. In further experimentation, *Vervliet et al.* (329) investigated pathologically induced calcium signalling within PACs. TLC-S was used to initiate intracellular calcium overload to test whether the application of exogenous BH4-Bcl-2 peptide also affects pathophysiological calcium signalling. Here, *Vervliet et al.* (329) clearly demonstrated protective effects by reducing cellular necrosis in addition to inhibiting cytosolic calcium levels in response to TLC-S. The findings of this ground-breaking study revealed for the first time that the BH4 domain of Bcl-2 can be utilised to reduce excessive RyR-mediated $Ca^{2+}$ release. In doing so, sheds light upon

the potential further use of innovative peptides as tools to combat pathology in the development of Acute Pancreatitis.

### 1.25 Pharmacological Tools as Potential Therapeutics

More commonly than the use of peptides as potential therapeutic strategies, pharmacological agents were of greater interest. The functions of these pharmacological compounds range greatly depending on their structure. *Trans*-NED-19 is a very well-known NAADP non-competitive antagonist (175,333,334). The *trans* form of NED-19 is much more powerful and potent than the *cis* structure (333). Trans-NED-19 is cell permeant and so does not require any additional procedures for entry into the cell. NED-19 inhibits the NAADP binding along with NAADP-mediated $Ca^{2+}$ release by binding to receptors of NAADP (333). Ever since its discovery, it has been widely used to aid the investigation of NAADP-mediated $Ca^{2+}$ signalling (175,333–335).

NED-19 inhibits NAADP mediated calcium signalling however, a hypothesis over the years has been to target inhibition of calcium entry within cells. Due to the correlation and involvement of calcium entry in pathological calcium signalling, this area of study has been of extreme importance. Therefore, pharmacological compounds which target specific CRAC channels have been developed such as GSK-7975A and CM-4620. GSK-7975A is a compound developed by GlaxoSmithKline and blocks Orai calcium entry channels (34,297,336–338). This drug is extremely effective in blocking store-operated $Ca^{2+}$ entry in a variety of cell types (34,297,337,338). This selective inhibition of Orai CRAC channels using GSK-7975A has made this drug an extremely useful diagnostic tool in investigating pathological calcium signalling. Not only are compounds such as GSK-7975A useful investigative tools but can also be applied as therapeutic compounds. An example of this is the selective Orai1 inhibitor, CM4620 (170,339). This compound, made by CalciMedica displays selectivity for Orai1-containin channels and structurally contains a pyrazine core. This compound has showed significant inhibition of SOCE which advanced the

drug into Phase 1 clinical trials in 2017 (296,339). The drug then moved onto Phase 2 clinical trials in 2018 in patients with predicted moderate to severe acute pancreatitis. The results of which have been very promising, demonstrating better tolerance to solid foods, less persistent systemic inflammatory response syndrome and reduced hospitalisation when compared to standard of care alone (340,341). Although this open-label study was not able to provide statistical significances due to its small sample sizes, it does provide favourable reassurance to the safety and efficacy of CM4620, which is now commercially known as Auxora. Auxora will now take part in an improved, larger randomised, double-blind Phase 2b study to further investigate the effectiveness of Orai inhibition during the treatment of acute pancreatitis (340).

### 1.26 Other Potential Therapeutics

In addition to the use of peptides and pharmacological agents as potential therapeutics, some interest has been made regarding the use of sugars to reduce the loss of ATP production in cells during the development of acute pancreatitis (276). During the application of acute pancreatitis-inducing agents on PACs, glucose metabolism was reduced. As a consequence, this reduction led to diminished ATP synthesis which inherently led to cellular necrosis. A ground-breaking study by *Peng et al.* (276) demonstrated substantially reduced cellular injury induced by pathological agents due to the additional application of galactose. Galactose is known to enter cells via the same transporters as glucose but galactose is converted to glucose-6-phosphate by several enzymes whilst not involving hexokinases, unlike glucose (342). Furthermore, the application of pathological agents such as bile acids demonstrated inhibition of hexokinases, suggesting these enzymes play a vital role in ATP depletion during the development of acute pancreatitis. Due to this non-reliance upon hexokinases, galactose can protect cells against ATP depletion and necrosis during cellular stress (35,276,342). Therefore, galactose has been proposed as potential therapeutic and preventative measure against the development of acute pancreatitis which requires further investigation.

### 1.27 Aims and Objectives

This study consists of 2 distinct aims of investigation:

**Aim 1: Investigation of BH4 domain Bcl-2 protein derived peptides on mitochondria and cell survival in exocrine pancreas**

Previous work has highlighted the greatly beneficial protective effects of the BH4-Bcl-2 peptide family. During studies of PACs, BH4-Bcl-2 peptides demonstrated significant protection against pathology. However, these studies focussed upon cytosolic calcium events within PACs purely utilising BH4-Bcl-2 peptides. Therefore, this chapter aims to further study these BH4-Bcl-2 peptides during mitochondrial pathology within PACs. As there are significant downstream effects of the pathological cytosolic calcium signalling, it is of interest to monitor any positive protective effects on mitochondria caused by the application of BH4-Bcl-2. Additionally, it is aimed to reduce the concentration of BH4-Bcl-2 utilised whilst in combination with galactose in order to reduce the concentration of DMSO being applied to cells whilst also aiming to achieve similar protective effects compared to using higher concentrations of BH4-Bcl-2. The use of 50µM BH4-Bcl-2 subject cells to a DMSO concentration of 0.167% whilst the application of 25µM BH4-Bcl-2 would reduce this to 0.084%. This is vitally important to limit the risk of cellular damage due to the use of high DMSO concentrations.

**Objectives:**

1. Investigate effects of BH4-Bcl-2 and galactose upon mitochondrial calcium levels during pathological stimuli.
2. Investigate potential protective effects of lower concentrations of BH4-Bcl-2 in addition to combinatory use of BH4-Bcl-2 and D-galactose by carrying out necrosis assays.

**Aim 2: Investigation of potential protective effects of inhibitors of calcium entry**

Physiological and pathophysiological calcium signalling within PACs and PSCs involves a complex mechanism usually comprising of initial calcium release from internal stores followed by calcium entry from extracellular stores. Therefore, these signalling mechanisms pose potential areas for therapeutic intervention. Furthermore, inhibition of any of these areas may also provide answers to theories relating to underpinning mechanisms. The use of inhibitors offers a promising route for therapeutic intervention. Examples of these inhibitors are NED-19, an antagonist of NAADP, and CM4620, a CRAC channel inhibitor. These compounds could be extremely important in the battle against the development of acute pancreatitis by preventing pathological calcium signalling in PACs and PSCs.

**Objectives:**

1. Investigate the effects of the calcium entry inhibitor, CM4620, upon cytosolic calcium signalling during the application of physiological concentrations ACh.
2. Assess the effectiveness of NED-19 inhibition upon calcium entry within PACs.
3. Compare the effectiveness of NED-19 as an inhibitor of calcium entry to other well-known inhibitors of calcium entry in addition to assessing the effects of combinatory treatments of NED-19 and these other inhibitory compounds.

# Chapter 2

# Materials and Methods

## 2.1 List of Reagents

Asparaginase, obtained from Abcam, UK.

Calcium chloride ($CaCl_2$) obtained from Fluka, UK.

CM4620 was gifted by CalciMedica, California, USA.

Cyclopiazonic Acid (CPA), obtained from Merck Millipore, UK.

Fluo-4-AM, obtained from Molecular Probes, UK.

GSK-7975A was a gift from GlaxoSmithKline, Stevenage, UK.

NED-19, obtained from TOCRIS Europe.

Nuclear Green DCS1, obtained from Abcam, UK.

Potassium Chloride (KCl), obtained from Calbiochem, UK.

Propidium iodide (PI), obtained from Thermo Fisher Scientific, USA.

Rhod-2-AM, obtained from Thermo Fisher Scientific, UK.

Thapsigargin was obtained from Calbiochem, Merck Millipore, Watford UK.

All chemicals that were not stated above were supplied by Sigma-Aldrich, UK. All stock solutions were appropriately made in either sodium HEPES solution without supplemental $Ca^{2+}$, DMSO or water. This depended upon the solubility of the compounds being dissolved and stored at manufacturers' recommended storage conditions at either -80°C, -20°C, 4°C or at room temperature. Additionally, solutions were stored away from light if required. Prior to experimentation, unless stated, all final solutions were prepared in sodium HEPES solution supplemented with 1 mM $Ca^{2+}$.

## 2.2 Preparations of Buffers, Solutions, Dyes and Compounds

For the experiments involved in the project, a number of buffers and solutions were required. Firstly, the preparation of a Sodium HEPES buffer was needed to store and incubate the isolated cells.

### 2.2.1 Sodium HEPES Buffer

The Sodium HEPES Buffer was made by combining the compounds shown in Table 2.1. This buffer solution was utilised during the isolation of pancreatic tissue and cells. It was also used in all perfusion experiments as a medium to bathe the cells without inducing unwanted necrosis. Before making the $Na^+$ HEPES buffer, a stock of sodium hydroxide (5 M concentration) was made by dissolving NaOH pellets in MilliQ water. All equipment was washed thoroughly with MilliQ water.

All of the ingredients indicated in Table 2.1 were weighed and dissolved into MilliQ water. Following these additions, a pH meter was then used to measure the pH of the buffer. If the pH was not 7.2 then NaOH was added dropwise until the pH was suitable. The buffer was then transferred to a volumetric flask. Additional MilliQ water was added until the desired volume was made. The osmolarity of the newly made buffer was calculated by taking 55 µL of the buffer and using a Cryometric Osmometer. To be viable for experimental use, the $Na^+$ HEPES buffer must have an osmolarity of 290-300 mOsm/L and was stored at 4$^o$C.

***Table 2.1 Compounds required to make $Na^+$ HEPES Buffer***

| Compounds | Concentration (mM) |
|---|---|
| NaCl | 140 |
| KCl | 4.7 |
| $MgCl_2$ | 1 |
| HEPES | 10 |
| D-Glucose | 10 |
| $CaCl_2$ | 1 |

### 2.2.2 Collagenase Solution

A collagenase solution was required to breakdown the collagen fibres between cells during tissue isolation. The Type V Collagenase 25 U/mL stock was made. The final solution was then aliquoted into 1 ml aliquots which were then ready to be used during pancreatic tissue isolations. The aliquots containing the collagenase solutions were frozen at -20$^o$C until required.

### 2.2.3 Fluo-4-AM

A supply of 50 µg of Fluo-4-AM was dissolved into 25 µL of DMSO producing a 2 mM stock. This 2 mM solution was vortexed adequately, aliquoted into 5uL aliquots and stored in aluminium foil at -20$^o$C. During its use, this Fluo-4-AM stock was used to produce a final 10 µM working concentration.

### 2.2.4 Rhod-2-AM

A supply of 50 µg of was dissolved into 25 µL of DMSO to produce a 2 mM stock solution. This 2 mM solution was vortexed adequately, aliquoted into 10 µL aliquots

and stored in aluminium foil at -20$^{o}$C. During its use, this Fluo-4-AM stock was used to produce a final 20 µM working concentration.

### 2.2.5 Asparaginase Solution

Asparaginase stock solution with a concentration of 5000 IU/ml was prepared in water. To create the asparaginase concentrations required during the experiments, standard concentrations of Asparaginase were required. The stock vial contained 2400 Units. This was diluted to give 200Units per 1 ml of buffer. The concentrations of asparaginase were either 200Units or 400Units, therefore, appropriate dilutions were made in order to produce the correct final concentration.

### 2.2.6 Bradykinin Solution

To produce a 1 nM solution of Bradykinin that was used in the experiments, a dilution series was made from a 0.5 mM stock of 10 µL.

### 2.2.7 Calcium Chloride

Calcium chloride ($CaCl_2$) obtained from Fluka, UK was provided with a stock concentration of 1 M.

### 2.2.8 Cyclopiazonic Acid

Cyclopiazonic Acid (CPA) was stored at -20$^{o}$C in 40 µL aliquots with a stock concentration of 20mM dissolved in DMSO.

### 2.2.9 BH4-Bcl-2

The peptide was provided in powdered form and >80% purity. A stock concentration of 30 mM was dissolved in DMSO. The BH4-Bcl-2 peptide was

provided by Prof. Geert Bultynck and Dr. Tim Vervliet with additional peptide obtained from GenScript Biotech, UK.

### 2.2.10 NED-19 Solution

The concentration of NED-19 that was utilised in experiments was 100 µM. The original stock of NED-19 was 10 mg of solidified compound which required the addition of 190 µL of DMSO to create a 100 mM stock solution. This was then diluted to give a desired volume of the required concentration.

### 2.2.11 ACh Solution

ACh was produced with a stock concentration of 10 mM in MilliQ water. To produce the 3 concentrations of ACh that were required for experiments a dilution series was made in sodium HEPES buffer. This made it possible to create solutions of ACh with concentrations of 10 nM, 20 nM and 1 µM.

### 2.2.12 CCK Solution

A stock concentration of Cholecystokinin (CCK) was produced with a stock concentration of 5 µM. To produce a 5 pM solution of CCK that was used in the experiments, a dilution series was made from a solidified stock of CCK. 250 µg of CCK were initially dissolved into 43.73 mls of water. This then produced a 5 µM stock solution which was used as part of a dilution series.

### 2.2.13 GSK-7975A

GSK-7975A was dissolved in DMSO to produce a stock concentration of 10 mM.

### 2.2.14 Propidium Iodide

Propidium Iodide (PI), a stock solution with a concentration of 1 mg/mL was purchased.

### 2.2.15 Nuclear Green DCS1

Sufficient volume of Nuclear Green DCS1 was used to create a final working concentration of 5 μM.

### 2.2.16 Sodium Hydroxide

Sodium hydroxide (NaOH), obtained from Sigma-Aldrich, UK. Stored at room temperature with a stock concentration of 5 M dissolved in water.

### 2.2.17 Thapsigargin

Prepared in DMSO at stock concentration of 10 mM and was aliquoted and stored at -20 °C.

### 2.2.18 Taurolithocholic Acid 3-Sulphate

Taurolithocholic Acid 3-Sulphate (TLC-S) was dissolved in water and made a stock concentration of 2 M. During this, the water required warming to aid the dissolving the solid form of TLC-S prior to diluting it to a working concentration.

## 2.3 Tissue Isolation

In order to carry out experiments on pancreatic acinar and stellate cells it was necessary to use C57BL/6J mice obtained from Charles River UK Ltd. These mice were selected for use as they are a widely used general purpose strain. All mice which were used were male and were at least 6 weeks old but there was no age control. In accordance with the Animal Scientific Procedures Act 1986 and approval from the Ethical Review Committee of Cardiff University, the mice were sacrificed under schedule 1 by cervical dislocation.

### 2.3.1 Dissection

Following the Schedule 1 procedure using dislocation of the neck, the cadaver was then dissected. The skin was cut using on the left posterior flank to expose the inner abdomen. The inner membrane was then cut. Following this, the spleen was identified in order to locate the pancreas. The pancreas was then detached from the spleen and gut. The pancreas was then transferred to a weighing boat containing Na+ HEPES buffer prior to collagenase digestion. Once removed, the pancreas was used immediately for experimental work. The remainder of the cadaver was stored in a freezer at -20$^{o}$C before disposal.

### 2.3.2 Pancreatic Acinar Cells

Following dissection, the pancreas was washed twice in $Na^{+}$ HEPES buffer and then injected with collagenase solution using a 1 ml 29G insulin needle. The pancreas was then incubated in a 37$^{o}$C shaker water bath for 6 minutes and 30 seconds. The collagenase solution was then removed from the pancreas which in turn was transferred to a new container. The pancreas then underwent repeated pipetting to separate the cells from one another. The cells were then centrifuged using a workbench centrifuge whilst the remnants of the pancreas were discarded. The supernatant from centrifugation was discarded with the pellet of cells being resuspended in fresh $Na^{+}$ HEPES buffer.

### 2.3.3 Pancreatic Lobules

As with the isolation of PACs following dissection, the pancreas was washed twice in $Na^{+}$ HEPES buffer and then injected with collagenase solution using a 1 ml 29G insulin needle. As stellate cells are only found in pancreatic lobules, the pancreas was incubated in a 37$^{o}$C water bath for a slightly shorter time of 6 minutes and 5 seconds. The collagenase solution was then removed from the pancreas which in turn was transferred to a new container. The pancreas then underwent repeated pipetting to separate the pancreatic tissue in order to leave multiple lobules of cells.

To carry out this procedure a large pipette tip, which was larger than that used for the isolation of acinar cells, was used in order to isolate small lobules of pancreatic tissue. The cells were then centrifuged using a workbench centrifuge whilst the remnants of the pancreas were discarded. The supernatant from centrifugation was also discarded with the pellet of cells being resuspended in fresh $Na^+$ HEPES buffer.

## 2.4 Intracellular Calcium Measurements

### 2.4.1 Fluorescent Dyes

In order to obtain intracellular calcium measurements, the cells under investigation required loading with fluorescent dyes. Each experiment and dye required a specific loading time and concentration depending on the purpose of the experiment which are described in the following sections. Fluo-4-AM is also in its esterified form which are hydrophobic and uncharged molecules. These properties enable the dye to cross the cell membrane of the cell. Within the cytosol of the cell, the acetoxymethyl (AM) esters of Fluo-4-AM are removed by esterase enzymes in the cell. This removal restores the free acidic forms of the fluorescent Fluo-4 which is now trapped within the cell.

### 2.4.2 Intracellular Calcium in Pancreatic Acinar Cells

In order to measure intracellular cytosolic calcium levels of PACs, the fluorescent probe Fluo-4-AM was used. The cells were prepared for calcium imaging by loading the samples with 5 µM Fluo-4-AM following the manufacturer's instruction for a duration of 45 minutes at room temperature following tissue isolation. The cells were then washed and re-suspended in an extracellular solution of $Na^+$ HEPES Buffer containing 1 mM $Ca^{2+}$ prior to imaging described in 2.4.4.

### 2.4.3 Pancreatic Stellate Cells

The fluorescent probe Fluo-4-AM was also utilised to measure intracellular cytosolic calcium levels of PSCs. However, due to differing cell types, the preparatory protocol was also different. The tissue was prepared for calcium imaging by loading the samples with 5 µM Fluo-4-AM following the manufacturer's instruction for a duration of 35 minutes at room temperature following tissue isolation. This reduced loading time compared to isolated PACs was needed as the uptake of Fluo-4-AM in PSCs is greater than PACS, therefore, if a shorter loading time is used, PSCs fluoresce brighter and so can be identified in addition to their distinct shape. The cells were then washed and re-suspended in an extracellular solution of $Na^+$ HEPES Buffer prior to imaging described in 2.2.4.

### 2.4.4 Cytosolic Calcium Imaging

Several different microscopes were utilised during the recording of intracellular calcium measurements throughout the project. The majority of recordings were taken using an Argon powered Leica TCS SPE confocal microscope demonstrated. An excitation laser at a wavelength of 488nm was required to activate Fluo-4-AM that the cells were previously loaded with as described in 2.4.2 and 2.4.3. During loading, the cells absorb the Fluo-4-AM. The excitation laser is set at a specific wavelength of 488nm to excite Fluo-4-AM. If calcium is present, Fluo-4-AM is then excited which emits light at an approximate wavelength of 506nm. This light can then be detected and quantified to give a relative intensity of the level of calcium present within the target cell.

The isolated cells were attached to coverslips within a perfusion chamber placed on the microscope stage for investigation. A perfusion system was used to alter the solutions applied to the cells. This perfusion system consists of a number of syringes above the microscope that are connected by tubing to feed into the perfusion chamber of the microscope stage. The solutions/compounds of interest are stored in the syringes during an experiment. When required, each of the syringes could be

opened or closed to apply the compounds to the cells under investigation. With the assistance of gravity, the solution flows through the tubing into the perfusion chamber and bathes the cells.

Each experiment was designed individually and used different timing intervals for the application of compounds and washing. The results of the calcium imaging are stored and analysed using the Leica Imaging Software on a connected PC.

### 2.4.5 Mitochondrial Calcium Imaging in PACs

To measure mitochondrial calcium in PACs the fluorescent probe Rhod-2-AM was used. The cells were prepared for calcium imaging by loading the samples with 10 µM Rhod-2-AM following the manufacturer's instruction for a duration of 48 minutes at a temperature of 30$^{o}$C following tissue isolation. The cells were then washed and re-suspended in an extracellular solution of $Na^{+}$ HEPES Buffer containing 1 mM $Ca^{2+}$ prior to imaging. Due to the overall positive charge of the AM esters within the dye's structure, the molecule is able to translocate into the cellular mitochondria. Whilst the majority of the de-esterified Rhod-2 is located in the mitochondria, there is still a proportion of the dye withheld in the cytosol. Recordings were taken using an Argon powered Leica TCS SPE confocal microscope along with an excitation laser of 532nm was utilised. A wavelength of 535nm is sufficient to excite the Rhod-2 molecule which in turn emits peak wavelengths of 581nm.

### 2.4.6 Intracellular Calcium Measurements

During the investigation of intracellular calcium, the cells under investigation were located and brought within optical focus of the microscope. Regions of Interest (ROI) were drawn to analyse the calcium levels within cells. Firstly, an ROI was placed to measure the background levels of fluorescence, this ROI was then subtracted from the following ROIs containing cellular locations. Secondly for cytosolic calcium measurements, ROIs were drawn on the intended locations within

PACs or PSCs depending on the experiment. For mitochondrial calcium measurements, ROIs were drawn separately on cytosolic areas of PACs, including the nucleus whilst other ROIs placed upon visible mitochondria as shown in Fig. 3.1A.

### 2.4.7 Calcium Entry Protocol

Calcium entry into cells is measured using a specific protocol that includes the depletion of intracellular calcium stores. The cells are bathed in a calcium free solution. 10 µM CPA was then added to cells to deplete intracellular calcium stores which is characterised as a sudden increase and gradual decline of cytosolic calcium. During the whole experiment, 10 µM CPA was continuously applied which prevented the uptake of calcium into the stores, therefore, calcium entry and extrusion can be monitored. The application of 5 mM external calcium induces the entry of calcium into the cell. Prior to this application of external calcium, the cells were incubated for 15 mins with the proposed treatments, however, this incubation was not recorded to limit the stress applied to the target cells. The constant application of high energy laser wavelengths of light increases the production of ROS within cells as well as inducing photobleaching of the loaded fluorescent dye. Both of these consequences could significantly affect the outcome of the experiment; therefore, the minimum time of recordings were made. Once external calcium was applied and the maximal intracellular calcium level was obtained, the external calcium was removed. This removal of external calcium prompts its extrusion from the cytosol of cells.

## 2.5 Testing Protective Agents

### 2.5.1 Testing of NED-19

The experiments which investigated the inhibitory effects of 100 µM NED-19 on calcium entry included the application of 10 µM CPA in a calcium-free solution. Following the stabilisation of the intracellular calcium levels a calcium containing solution was then applied to evoke calcium entry. When investigating NED-19 and GSK-7975A, the calcium containing solution would also contain these compounds. The cells were pre-incubated for 10 minutes with the inhibitory compounds prior to the application of extracellular calcium.

### 2.5.2 Testing of BH4-Bcl-2 Peptide

The experiments which investigated the protective effects of BH4-Bcl-2 during mitochondria signalling included the application of 200 µM TLC-S or 5pM CCK in order to trigger a calcium response. When investigating the peptide, the cells were pre-incubated for 15 minutes with 50 µM of the peptide prior to the start of the experiment. The experiments were all carried out with a calcium containing $Na^+$ HEPES solution. Furthermore, during experiments which also investigated the effects of galactose, the cells were also pre-incubated for the same duration with 1 mM D-galactose.

## 2.6 Cellular Necrosis

### 2.6.1 Isolated PAC Necrosis

Following the isolation of cells according to 2.3.2, cells were divided into control and test sample sets during each experiment. The control set of cells acted as a negative control which only contained $Na^+$-HEPES buffer solution and 1 mM $Ca^{2+}$. A positive control was created by adding either 400 Units of Asparaginase or 200 µM TLC-S. During the testing of potential protective agents, cells were incubated for 15mins with protective agent prior to the addition of a necrosis inducing agent, such as Asparaginase or TLC-S. All samples (excluding negative control) were incubated for 2 hours with necrosis inducing agents at room temperature. In order

to allow each sample to be imaged at similar timings following incubations, the incubations of each sample were staggered and imaged at timed intervals which allowed sufficient time for imaging. 30 minutes prior to imaging, 5 µM of Nuclear Green DCS1 to each sample. Cells were then imaged using an inverted Zeiss spinning disc confocal microscope (20x air objective). The Nuclear Green DCS1 was excited using a 488nm laser and the emitted light was to differentiate between necrotic and healthy PACs. A photo was then taken of every group of cells until at least 100 cells were recorded. The total number of necrotic and healthy cells were analysed and counted. The numbers of necrotic cells are presented as a percentage of the total number of cells counted ± SEM.

### 2.6.2 Isolated Pancreatic Lobule Necrosis

Following the isolation of cells according to 2.3.3, cells were divided into control and test sample sets during each experiment. The control set of cells acted as a negative control which only contained $Na^+$-HEPES buffer solution and 1 mM $Ca^{2+}$. A positive control was created by adding 400 µM TLC-S. During the testing of potential protective agents, cells were incubated for 15 minutes with protective agent prior to the addition of the necrosis inducing agent, TLC-S. All samples (excluding negative control) were incubated for 2 hours with necrosis inducing agents at room temperature. In order to allow each sample to be imaged at similar timings following incubations, the incubations of each sample were staggered and imaged at timed intervals which allowed sufficient time for imaging. This sample was then loaded with a working concentration of 5 µM of Fluo-4-AM 30 minutes before the end of the 2-hour incubation. Additionally, 10 minutes prior to the end of the incubation 2 µL of Hoechst and 2 µL of Propidium Iodide was added to the sample. All samples were then analysed using a Leica Confocal Microscope. Clusters of cells were analysed with sequential reads of 3 measuring channels with excitation lasers of 534nm, 488nm and 405nm wavelengths. During the analysis of pancreatic lobules, PSCs were firstly identified by their distinct shape and bright Fluo-4 staining. To identify necrotic PSCs the combinatory use of Hoechst and PI staining

alongside Fluo-4 staining were used. Once identified from Fluo-4 staining, the cell's nucleus was assessed with Hoechst labelling which confirmed the number of cells present in the area and separated the nuclei of local PACs. Finally, it was assessed whether the target cell was PI stained positive whilst also retaining a cellular membrane, if this was the case, the cell was counted as a necrotic cell. If the cell was not identified as PI positive stained, the cell was counted as a viable, living cell. 20 to 30 images were captured per condition and the number of viable cells and necrotic cells (propidium iodide positive staining) were counted.

### 2.7 Statistical Analysis

The data shown are presented with mean values along with ±SEM. Any statistical significance and p-values were calculated using two-tailed unpaired t-tests or a One-Way ANOVA. A p-value that was considered significant was $p < 0.05$. MATLAB, Excel and PRISM GraphPad was utilised to perform trace analysis whilst PRISM GraphPad was used to carry out statistical analysis on data. Where statistical analysis was carried out, the P-Value of each test will be represented as: * = P-Value <0.05, ** = P-Value <0.01, *** = P-Value <0.001, **** = P-Value <0.0001.

# Chapter 3 – Results

# The Protective Effects of BH4-Bcl-2 in PACs and PSCs

### 3.1 Introduction

For some time, interest in the use of peptide-based therapeutics has been growing. The B-cell lymphoma 2 family of proteins are well known to play a crucial part in regulating apoptosis in cells (321,329,330). The differing domains within the Bcl-2 family also possess differing functions. The overexpression of Bcl-2 family proteins is heavily implicated in various cancers which triggered interest to utilise Bcl-2 proteins to treat some malignancies. Whilst some of the family members of proteins are antiapoptotic, some are proapoptotic such as BH3-only protein domains. As a consequence, the identification of specific Bcl-2 domains and the creating of corresponding cell permeable peptides has provided interesting results and potential anti-cancer therapeutics. Furthermore, recent studies have investigated these peptide domains in treating other pathological diseases other than cancer. Recently, it has emerged that Bcl-2 proteins are not only important in the regulation of apoptosis but are also key modulators of intracellular calcium signalling (171,322,326,329,330). Specifically, the BH4 domain derived peptide from the Bcl-2 family of proteins inhibits both pathological and physiological signalling mediated by either $IP_3Rs$ or RyRs (325,329).

Work carried out by Vervliet *et al.* (329) demonstrated drastically reduced cytosolic calcium levels during CCK and TLC-S induction. Vervliet *et al.* (329) tested the application of multiple Bcl-2 peptides, including BH4-Bcl-2, upon PACs during CCK and TLC-S induced calcium signalling. During which, it was shown that cytosolic calcium levels were significantly reduced due to the application of BH4-Bcl-2. However, due to the importance of mitochondrial calcium levels during cell survival, it was not known whether or not the derived BH4 peptide is also able to demonstrate protective effects in mitochondria calcium signalling.

### 3.2 Results - BH4-Bcl-2 and Physiological Mitochondrial Calcium Signalling

Initial experiments investigated the effects of BH4-Bcl-2 (BH4) upon physiological calcium signalling within mitochondria of isolated PACs. Not only do these results provide insight on the effects of BH4 but also confirms how mitochondria are affected by calcium signalling. The mitochondria of PACs as shown in Fig. 3.1 highlight the regions of interest within PACs loaded with Rhod-2-AM with mitochondria commonly forming a belt surrounding the granular area. The application of CCK to these cells usually produced a significant rise in mitochondrial calcium which slowly decreased over time towards the baseline, as shown in Fig. 3.2. The resulting average traces shown in Fig. 3.2 are compiled from the applications of BH4 and no peptide treatment. A scrambled form of the BH4 peptide was also assessed and showed no significant difference when compared to the control application of CCK, as shown in App. 1. Furthermore, during the recording of mitochondrial calcium, cytosolic calcium levels would also be recorded simultaneously within the same cells being studied. However, it was noticeable that the signalling patterns within the mitochondria were different compared to the cytosol. Cytosolic signalling seemed to include much more significant oscillatory signals but also did not yield a major signal overall as shown in App 2.

Quantifying these traces as shown in Fig. 3.2 yields a significantly reduced calcium signal in mitochondria within cells treated with the BH4 peptide. The application of 50 µM BH4 reduced the mean cytosolic calcium level from 2.277 ±0.2372 to 1.522 ±0.0721 with an observed p-value of 0.0110. However, this reduction was only observed in mitochondria with no difference seen between any datasets measuring cytosolic calcium highlighted in App. 2. Although this was an unexpected result, a p-value of 0.1727 confirmed there was no difference due to the application of BH4 which yielded a mean of 1.145 ±0.0380 compared to a control of 1.185 ±0.0277. Reasoning for this non-significance could be due to a lack of specificity for the Rhod-2-AM dye which was used and may be the cause of this discrepancy. On the other hand, the reduced mitochondrial calcium signal caused by BH4 was not shown when testing the BH4 scrambled peptide as shown in App. 1. There was no

statistical difference with a p-value of 0.3725 between the control dataset's mean of 2.277 ±0.2372 and a scrambled peptide's mean of 1.950 ±0.2261. Following these experiments testing physiological mitochondrial signalling, more interest is aimed towards pathophysiological signalling, therefore, future experimentation was planned to assess the effectiveness of BH4 upon pathophysiological stimulation.

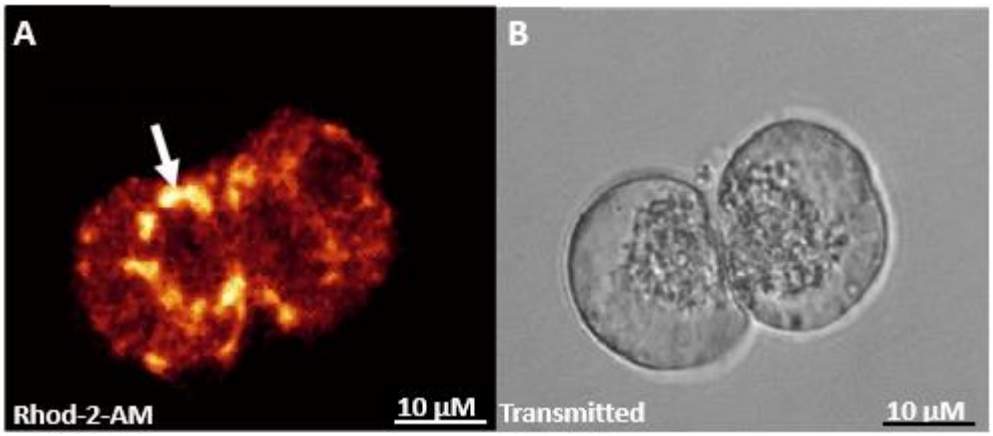



***Figure 3.1 Images of PACs during live cell imaging and recording.***

***(A)*** *Fluorescent image of isolated mouse PACs loaded with Rhod-2-AM. As indicated by arrow, mitochondrial areas within the cell can be seen during confocal imaging.* ***(B)*** *Transmitted light image of the same duplet of PACs shown in (A).*

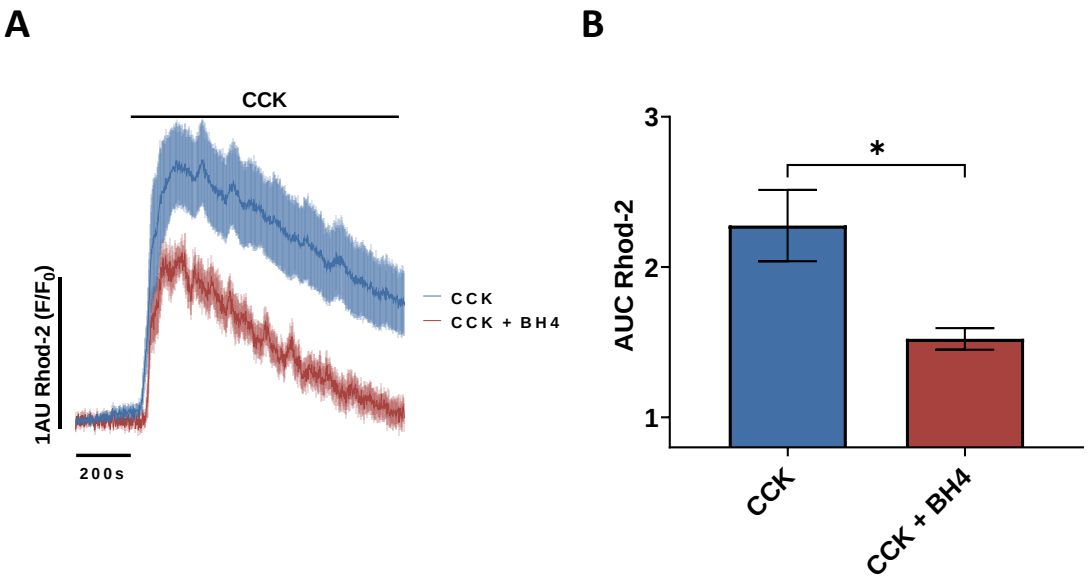



***Figure 3.2 Mitochondrial calcium analysis of isolated PACs in response to CCK.*** *Average mitochondrial calcium traces of isolated Rhod-2-AM loaded PACs in response to 5pM CCK stimulation.* ***(A)*** *Cells were either untreated controls with DMSO addition (n=12) or treated with 50 µM BH4-Bcl-2 peptide (n=10). Average traces were plotted with corresponding ±SEM values for mitochondrial traces. Cells were pre-incubated for 15mins with respective treatments and 5pM CCK was applied at 200 seconds. Buffer in every experiment contained 2mg/10mls of trypsin and chymotrypsin inhibitor.* ***(B)*** *The mitochondrial traces shown in (A) were quantified with total AUC.$s^{-1}$ values calculated from 200s to 1050s from start of experiment and plotted with ±SEM. A significant difference was observed between the control application of CCK with an average of 2.277 ±0.2372 and the application of 50 µM BH4-Bcl-2 peptide with an average value of 1.522 ±0.0721. An observed P-Value of 0.0110 was calculated between the two groups.*

### 3.3 Results - BH4-Bcl-2 reduces mitochondrial calcium responses to pathological stimuli

During testing of pathophysiological signalling, the bile acid TLC-S was utilised as a pathological stimulant. Average traces of PACs from multiple different combinations of treatments shown in Fig. 3.3 highlight a comparable story as found during physiological testing. The application of TLC-S induces a large, sustained response in mitochondria which generally slowly decreases over time. During this pathophysiological testing an additional set of treatments were created. These included the application of 1 mM Galactose (Gal) in addition to applying 1 mM galactose in combination with 50 µM BH4. This was due to the hypothesis that mitochondrial state and ATP regulation are vital for cell survival. As a consequence of this, the recent studies displaying the protective effects of Galactose during pancreatic pathology presented potential new avenues for treatments. Therefore, galactose was introduced to investigate its effects upon pathological mitochondrial calcium signalling.

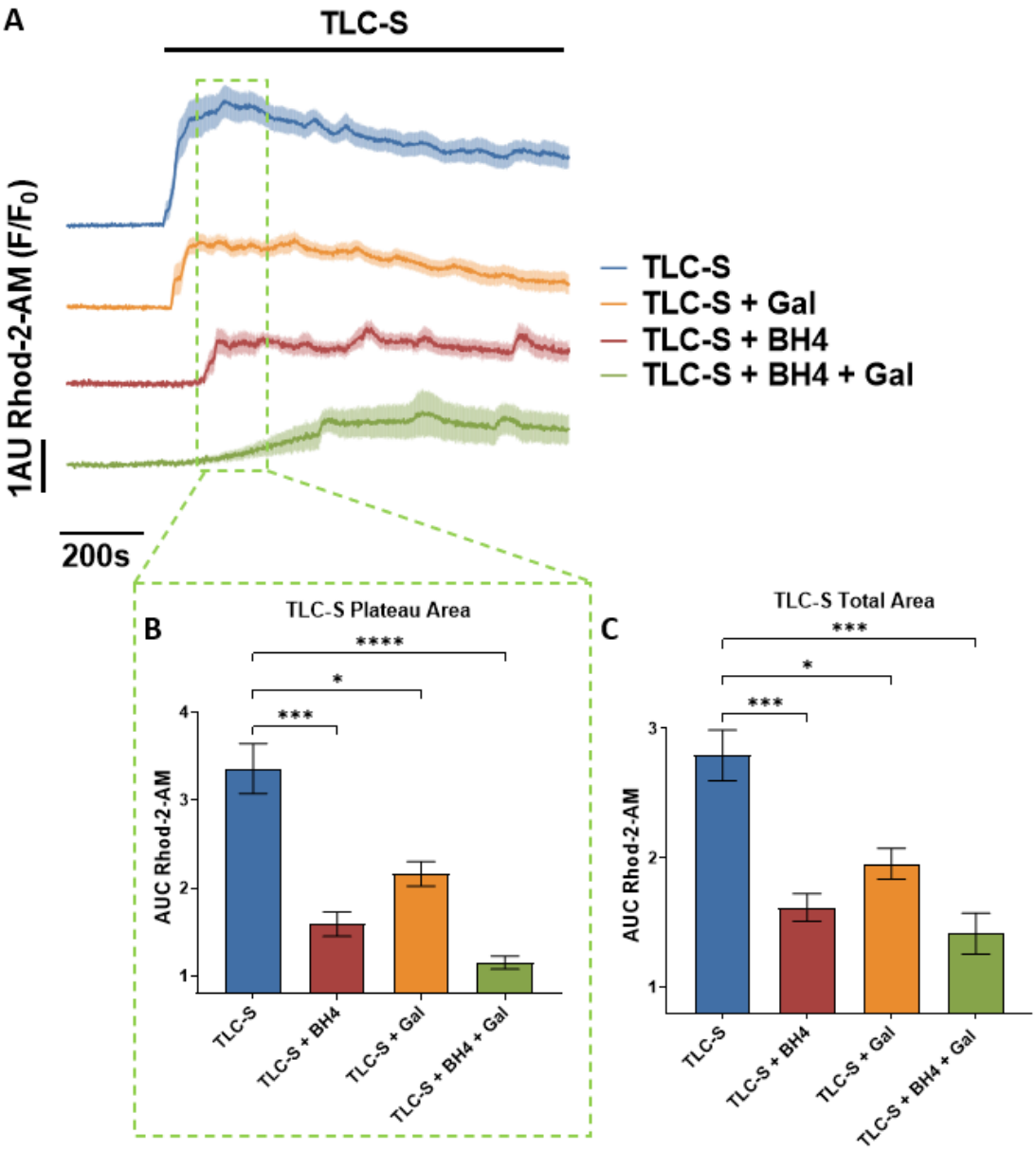
The resulting average traces displayed in Fig. 3.3 presents similar trace outlines for each experimental design with an exception for the combination treatment of BH4 and galactose. Visually, there is a noticeable difference between the traces as both cytosolic and mitochondrial traces present a slow incline of calcium levels in PACs treated with a combination of BH4 and galactose which is quantified and discussed later in Fig. 3.4. In addition to analysing the entirety of the mitochondrial traces, a small section of the traces near the initialisation of the signal was selected for further analysis. This was done to shed more light upon the part of the traces which are clearly different between each treatment group.

The analysis of the specified area in Fig. 3.3B highlighted significant differences between treatments. Upon comparison, analysis of the plateau region of the mitochondrial response in the control yielded significant differences between the control with a mean of 3.359 ±0.2842 and the application of BH4-Bcl-2 (1.588 ±0.1389), galactose (2.160 ±0.1404) and the combination of 50 µM BH4 and 1 mM galactose (1.150 ±0.0738). The comparison of the combination treatment and the

control yielded the greatest significant difference with a p-value of <0.0001. Comparison of the control and application of BH4 also produced a similar level of significance with a p-value of 0.0002 whilst 1 mM galactose produced a p-value of 0.0206 when compared with the control.

Further to this, analysis of the total area of the mitochondrial response demonstrated further differences, similar to those shown in Fig. 3.3B. Once more, all treatment groups demonstrated a significant reduction compared to the control mean of 2.793 ±0.1957. Correspondingly, the combination of BH4 and galactose yielded a mean of 1.414 ±0.1590 and a p-value of 0.0005. Treatment with BH4 also yielded a comparable level of difference with a p-value of 0.0008 whilst reducing the mean total AUC to 1.616. Finally, treatment with galactose produced the lowest reduction with a p-value of 0.0299 but did significantly reduce the total AUC of the response to 1.955.

Interestingly, the application of galactose also reduces the mitochondrial responses recorded to similar levels of BH4-Bcl-2. Furthermore, the combination application of BH4-Bcl-2 and galactose significantly reduced mitochondrial responses greater than the sole use of both of these treatments with the lowest reduced mean AUCs of the plateau of 1.150 ±0.0738 and of the total area with 1.414 ±0.1590. This potentially signifies that the inhibitory mechanisms of both treatments are most likely separate. The combination treatment induced a drastically prolonged response within mitochondria which is substantially different to that previously seen. As a consequence, this resulted in a greatly reduced response in the initial plateau phase of the response as well as the total area of the response. As the cytosolic comparisons did not yield comparable findings as the mitochondrial signalling, it was deemed that cytosolic measurements would not be analysed moving forward.


A
TLC-S
1AU Rhod-2-AM (F/F0)
200s
TLC-S
TLC-S + Gal
TLC-S + BH4
TLC-S + BH4 + Gal
B
TLC-S Plateau Area
AUC Rhod-2-AM
****
*
***
TLC-S
TLC-S + BH4
TLC-S + Gal
TLC-S + BH4 + Gal
C
TLC-S Total Area
AUC Rhod-2-AM
***
*
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TLC-S
TLC-S + BH4
TLC-S + Gal
TLC-S + BH4 + Gal

***Figure 3.3 Quantitative analysis of mitochondrial calcium of isolated PACs in response to TLC-S stimulation.***

*Average mitochondrial traces of isolated Rhod-2-AM loaded PACs in response to 200 µM TLC-S stimulation. Cells were either untreated controls with DMSO addition (n=15), treated with 50 µM BH4-Bcl2 peptide (n=7), treated with 1 mM Galactose with no peptide present (n=6) or a combination treatment of 50 µM of a BH4-Bcl2 peptide and 1 mM Galactose (n=5). Average traces were plotted with corresponding ±SEM values for mitochondrial traces shown in* ***(A)****. The entirety of the traces in (A) were analysed to determine total area of responses whilst the highlighted boxes indicate the region analysed to indicate difference within the plateau areas of each trace. Cells were pre-incubated for 15mins with respective treatments and 200 µM TLC-S was applied at 200 seconds. Buffer in every experiment contained 2mg/10mls of trypsin and chymotrypsin inhibitor. The average AUC.s$^{-1}$ values for the peak plateau area of responses were quantified in* ***(B)*** *for mitochondrial traces shown in (A). Average AUC.s$^{-1}$ values for this region was calculated from 350s to 600s from the start of recording and plotted with ±SEM. Statistical differences were only observed when comparing mitochondrial traces. There was a statistical difference between the Control dataset and 50 µM BH4 with a P-Value of 0.009 whilst in the combination treatment of 50 µM BH4 and 1 mM Gal showed a P-Value <0.0001. Furthermore, there was a significant difference seen between the combination treatment 50 µM BH4 and 1mM Gal with the single treatment of 1 mM Gal with a P-Value of 0.0315. The average AUC.s$^{-1}$ values for the total area of responses were quantified in* ***(C)*** *for mitochondrial traces shown in (a). Total AUC.s$^{-1}$ values were calculated from 200s to 1050s from start of experiment and plotted with ±SEM. Significant differences were observed only between mitochondrial traces. Again, there was a statistical difference between the Control dataset and 50 µM BH4 in addition to the combination treatment of 50 µM BH4 and 1 mM Gal with an observed P-Value of 0.0003. Furthermore, there was difference shown between the 1 mM Gal treatment and Control with a P-Value of 0.0337. (* = P-Value <0.05, ** = P-Value <0.01, *** = P-Value <0.001, **** = P-Value <0.0001).*

In addition to reduced areas of mitochondrial responses due to BH4-Bcl-2 and galactose there is a noticeable difference in the rate of increase of responses. This led to the quantified rate data shown in Fig. 3.4. The datasets have been quantified in two different aspects, firstly the initial rate of increase during responses shows a significant reduction due to the application of galactose (0.0195 $F/F_0.s^{-1}$ ±0.0023) and the combination treatment of BH4-Bcl-2 and galactose (0.003 $F/F_0.s^{-1}$ ±0.0006). The combination treatment demonstrated a significant difference compared to the control initial rate with a p-value of 0.0012. However, the singular use of BH4-Bcl-2 did not significantly affect the rate of increase during the mitochondrial response with BH4-Bcl-2 producing an initial rate of 0.033 $F/F_0.s^{-1}$ ±0.0068 compared to a control value of 0.0432 $F/F_0.s^{-1}$ ±0.0059. Furthermore, the addition of BH4-Bcl-2 to the galactose treatment also yielded a significantly reduced initial rate when compared to the sole use of galactose with a p-value of 0.0442.

The uninhibited sudden increase in mitochondrial calcium may be more damaging to the mitochondrial membrane compared to a higher amplitude during the prolonged calcium response. Therefore, it is beneficial to consider the combinatory use of BH4-Bcl-2 and galactose which significantly reduced the initial rate. Secondly, analysis of the time taken to reach ½ maximal amplitude shown in Fig. 3.4B provided some differing results to the initial rate as this parameter also considers the amplitude of the mitochondrial response. Here, it highlighted no difference in time due to the application of galactose with a p-value of 0.8333, however, the combination treatment of BH4-Bcl-2 and galactose yielded the greatest increase in time required to reach half maximal amplitude. The combination treatment was significantly different to the control dataset with a p-value of <0.0001 and an average difference of 252.8 s. Additionally, the combination treatment also demonstrated significant difference compared to the sole applications of 50 µM BH4 and 1 mM of galactose with p-values of <0.0001. Therefore, not only does the combination treatment reduce the initial rate it also considerably delays the overall response which may propose protective effects for the integrity of the mitochondrial membrane and ATP production.

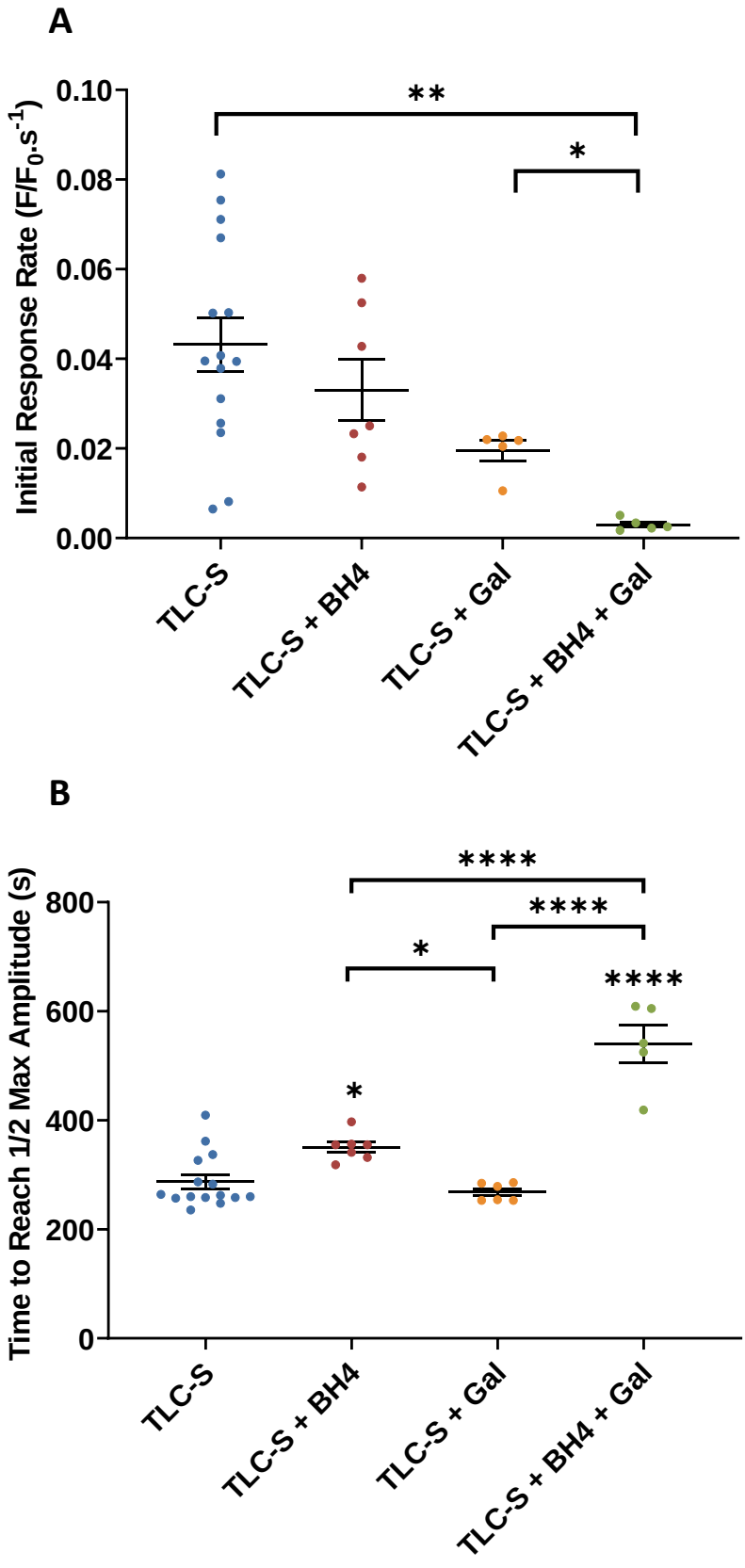

A
Initial Response Rate (F/F0.s-1)
0.10
0.08
0.06
0.04
0.02
0.00
**
*
TLC-S
TLC-S + BH4
TLC-S + Gal
TLC-S + BH4 + Gal
B
Time to Reach 1/2 Max Amplitude (s)
800
600
400
200
0
****
****
*
****
*
TLC-S
TLC-S + BH4
TLC-S + Gal
TLC-S + BH4 + Gal

***Figure 3.4 Rate of rise analysis of mitochondrial calcium traces of isolated PACs in response to TLC-S stimulation.***

*Rate of rise analysis of mitochondrial calcium traces of isolated PACs in response to 200 µM TLC-S stimulation. Cells were either untreated (Control) controls with DMSO addition (n=14), treated with 50 µM BH4-Bcl2 (BH4) peptide (n=7), treated with 1 mM Galactose (Gal) with no peptide present (n=6), a combination treatment of 50 µM of a BH4-Bcl2 peptide and 1 mM Galactose (n=5).* ***(A)*** *Initial rate analysis of traces. Traces for every experiment were analysed from the start of calcium increase to 50s after to quantify the initial rate of response once cells had responded to TLC-S application. Some statistical difference was seen between the combination treatment of BH4 + Gal and Control with a P-Value of 0.0012. Additionally, there was difference shown between the single use of BH4 and the combination BH4 + Gal treatment with a P-Value of 0.0442.* ***(B)*** *Half-max amplitude rate analysis. Traces were further analysed to calculate the time to reach half-maximal amplitude in each trace. Significant differences displayed directly above datasets show comparison to control with P-Values of 0.0281 and <0.0001 when analysing BH4 and BH4 + Gal respectively. Further differences were shown BH4 and Gal, P-Value = 0.0173, whilst combination of BH4 + Gal was significantly different to both single treatments of BH4 and Gal with a P-Value of <0.0001 in both comparisons. (* = P-Value <0.05, ** = P-Value <0.01, *** = P-Value <0.001, **** = P-Value <0.0001.)*

### 3.4 Results - BH4-Bcl2 grants protective effects against cell death in response to TLC-S

As a consequence of pathological calcium signalling within the cell, the cell becomes overloaded and so induces necrosis. This is a major step in the process of the development of acute pancreatitis. Therefore, it was investigated to assess the protective effects of BH4-Bcl-2 upon the levels of necrosis induced by pathological stimuli displayed in Fig. 3.5. This was carried out in a dose dependent manner during the application of the bile acid, TLC-S. It is clear that BH4-Bcl-2 grants some protective effects against the necrosis inducing agent as all of the treatments used were significantly different to the positive control application of TLC-S. However, none of the treatments restored necrosis levels entirely back to the negative control levels of 5.576% ±0.2876. The application of a high 50 μM dose of BH4 yielded a mean cell death percentage of 10.66% ±0.9947 which was significantly lower, with a p-value of <0.0001, than the positive control mean of 17.49% ±0.5347. Furthermore, this protective effect was also shown when the concentration of BH4-Bcl-2 was reduced to 20 μM but to an observable lesser degree. With an average cell death of 13.42% ±0.3135, the treatment of 20 μM BH4 significantly reduced cell death compared to the positive control with a p-value of 0.0004.

In a similar fashion to the previous experiments, the necrosis assays also included the combinatory use of BH4 alongside galactose. However, the sole application of galactose was not introduced as this has been studied in detail in previous publications (35,276). Instead, galactose was used alongside both concentrations of BH4, 20 μM and 50 μM. The addition of galactose to 50 μM of BH4 did not yield any further reduction in necrosis when compared to the sole application of 50 μM BH4. The combined treatment produced significantly reduced cell death with a mean of 9.405% ±0.6463 and when compared to the positive control, resulted in a p-value of <0.0001. However, interestingly, the combination of 20 μM BH4 with galactose yielded similar reductions of necrosis comparable to 50 μM of BH4 with galactose with a mean cell death of 10.49% ±0.8118. This resulted in no observable difference between the two combinatory treatments. As a consequence, there is no advantage

in applying a high 50 µM concentration of BH4 when in combination with 1 mM galactose. The use of this combination minimises the volume of DMSO used may prove to be a viable therapeutic strategy in the future whilst.

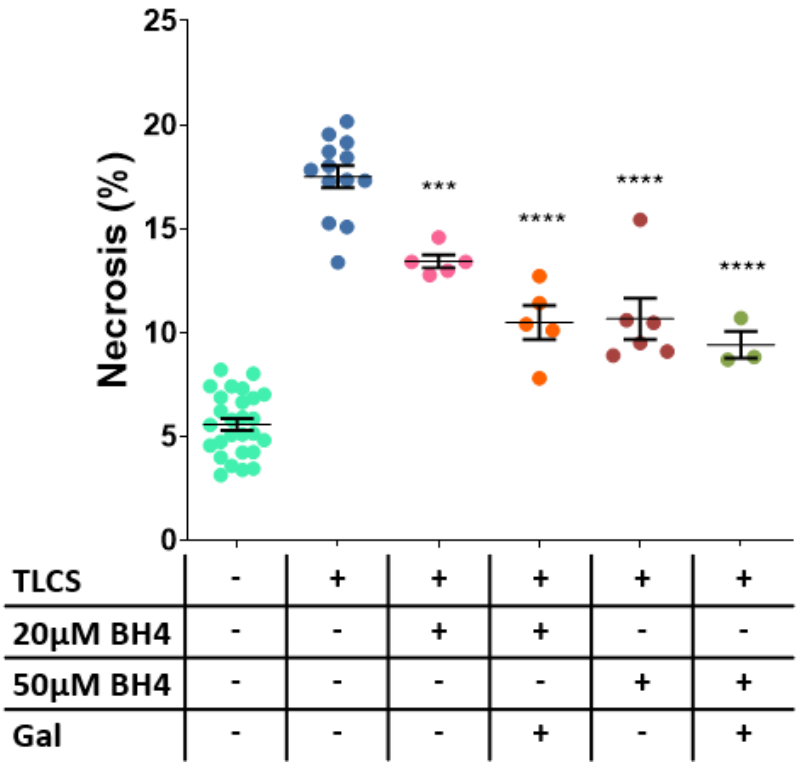



***Figure 3.5 Quantification of necrosis assays of isolated PACs displaying BH4-Bcl-2 protection in response to TLC-S.***

*Isolated PACS were treated under control conditions or by the incubation with varying concentrations of BH4-Bcl-2 (BH4) solely and in combination with 1 mM Galactose (Gal). Following pre-incubation of 15mins with treatments, pathological stimuli were applied to induce cell death over a 2-hour period, at which point necrosis was assessed after Nuclear Green DCS1 staining. The percentages of Nuclear Green DCS1-positive necrotic cells were assessed for each experimental condition. Necrosis was induced via the application of 200 µM TLC-S to induce necrosis. Each data point represents an independent repeat of the experiment, with the average values ±SEM displayed (* = P-Value <0.05, ** = P-Value <0.01, *** = P-Value <0.001, **** = P-Value <0.0001).*

### 3.5 Results - BH4-Bcl-2 provides protective effects in PSCs and PACs against pathological stimuli in pancreatic lobules.

The clear involvement of PSCs in the development of acute pancreatitis posed the question of whether the BH4-Bcl-2 peptide displays any protective effects within this cell type. Initially, it was planned to also study mitochondrial calcium levels within PSCs, however, insufficient Rhod-2-AM loading in pancreatic lobules resulted in the studying of cytosolic calcium levels in PSCs with Fluo-4-AM. It was vital to do this in order to assess the BH4 effects within PSCs which has never been studied before. As a result of investigating PSCs, PACs can also be monitored as the PSCs are located in isolated pancreatic lobules. Therefore, PACs are inherently found surrounding PSCs within lobules. As a further consequence to the use of pancreatic lobules, a higher concentration of pathological stimulus was required, therefore, it provided another opportunity to assess the effectiveness of BH4 protection in PACs.

The representative traces shown in Fig. 3.6 visibly show the differences between treatment conditions. Visibly, the BH4 treatment demonstrated almost gradual increases in cytosolic calcium in PSCs compared to the control with instantaneous spiking. Furthermore, the application of both BH4 and Gal yielded a vastly reduced overall induced signal which is quantified in Fig. 3.9C and Fig. 3.9E. Furthermore, this reduced overall calcium signal includes a much lower rate of rise. However, a very noticeable difference within these signals is made when compared to representative traces in Fig. 3.7. These representative traces in PACs, simultaneously recorded alongside PSCs show a remarkable increased sensitivity to TLC-S compared to PSCs. PACs clearly show a much greater increase in induced calcium signalling compared to PSCs during TLC-S stimulation. Furthermore, it is more evident the effects of the application of the treatments. All of the treatments demonstrate a reduced overall signal with lower amplitudes and shorter spiking throughout the application of TLC-S which are later quantified in Fig. 3.9. All of the traces shown in Fig. 3.6 and 3.7 were combined to form average traces displayed in Fig. 3.9A and Fig. 3.9B respectively. Again, this visually confirms the differences

between treatment groups. These traces were quantified with average AUCs being calculated within the analytical regions shown in Fig. 3.9.

The quantified results in Fig. 3.9 highlight reductions in cytosolic calcium levels in comparison with the treatments of BH4, galactose or the combination of the two. During analysis, two regions of the traces were quantified, a region near the start of the calcium response to demonstrate changes during the initial phase of the signal and a second area covering the overall signal. In PSCs, there were more differences shown between dataset during analysis of the initial region of the signal. As shown in Fig. 3.9C, significant reductions of cytosolic calcium were produced by the application of BH4, with a mean of 1.154 ±0.0683, Gal, with a mean of 1.119 ±0.0229, and the combination of BH4 and Gal, with a mean of 0.0150 ±0.0368. When these means were compared with a control mean of 1.450 ±0.0816, statistically significant p-values of 0.0054, 0.0007 and 0.0037 were calculated respectively. However, when analysing the total AUCs in PSCs, only two significant differences were observed. There was a significant difference between the control 1.466 ±0.0667 and Gal 1.204 ±0.0281 with a p-value of 0.0017 whilst comparison between control and the combination of 50 μM BH and 1 mM Gal, 1.237 ±0.0342, produced a p-value of 0.0105. Unlike what was seen during the initial area analysis, there was no significant difference recorded between the control and BH4, 1.324 ±0.0578, with a p-value of 0.2137. However, throughout these analyses there were no significant differences shown between any of the treatments themselves, therefore, the combination treatment did not provide any additional benefit in this case.

The quantification of traces recorded in PACs shown in Fig. 3.9D and Fig 3.9F provide differing results to those seen in PSCs. Firstly, the amplitudes and AUCs induced in PACs were much greater those recorded within PSCs. When taking a look at the initial AUCs, significant differences were observed when comparing the control dataset, 2.162, to the application of BH4, 1.344 ±0.0462, with a p-value of <0.0001, and to the application of the combination of BH4 and Gal, 1.398 ±0.0429, also with a p-value of <0.0001. When comparing the control against the application

of Gal, 1.614 ±0.0946, it produced a p-value of 0.0002. These findings were also extremely similar to those shown in Fig. 3.9F when the total AUCs were compared. Each treatment group demonstrated a significant reduction, all with p-values of <0.0001, when compared to the control. However, similar to the results seen in PSCs, there were no statistical differences observed between treatment groups, therefore, the combination of the BH4 and Gal did not produce any further beneficial reductions in cytosolic calcium concentrations.

Following cytosolic experiments, necrosis assays of pancreatic lobules were performed to assess whether the effects shown on cytosolic calcium transpire into protective effects against cell death. These results shown in Fig. 3.10 demonstrates similar protective effects of BH4 against necrosis in pancreatic lobules as seen in isolated PACs in Fig. 3.5. Since now, BH4 has been well established as a potential inhibitor of pathological signalling induced by agents such as TLC-S. The evidence in Fig. 3.9 suggests that BH4 may protect both PSCs and PACs against cell death as cytosolic calcium levels were significantly reduced as previously described. Therefore, we aimed to study the potential effects of BH4 upon cell death in pancreatic lobules. Compared to isolated PACs, the necrosis assays performed on pancreatic lobules utilised a higher concentration of TLC-S. As a consequence, the levels of necrosis in PACs were markedly higher compared to levels shown in Fig. 3.5. Again, 50 µM BH4 and a combination treatment of 50 µM BH4 and 1 mM Galactose were tested.

BH4 demonstrated significant protective effects in both PSCs and PACs by significantly reducing cell death in both cell types. In PSCs, no treatments were able to reduce necrosis levels low enough to return to control levels of 4.726% ±0.2947. However, both treatments did yield significant reductions compared to the positive control of 400 µM TLC-S with a mean of 18.44% ±0.4863. With a mean of 10.36% ±0.6622, the application of 50 µM BH4 produced a comparable p-value of <0.0001 as did the combination treatment of 50 µM BH4 and 1 mM Gal yielding a reduced mean cell death of 8.680% ±0.4863. However, there was no observable difference between the two treatments with a p-value of 0.6421.

The results in PACs shown in Fig. 3.10C tells a similar story as to that in PSCs and Fig. 3.10B. The mean cell death of the control dataset was 5.416% ±0.4288 with a positive control mean cell death of 23.89% ±0.4950, which is approximately 6% higher cell death than that observed in Fig. 3. 5. Again, both treatments provided significant reductions compared to 400 µM TLC-S with a mean of 16.25% ±0.1.312, the application of 50 µM BH4 produced a comparable p-value of <0.0001 as did the combination treatment of 50 µM BH4 and 1 mM Gal yielding a reduced mean cell death of 12.63% ±1.137. However, unlike the results seen in PSCs, there was a statistical difference between the two treatment groups with a p-value of 0.0364.

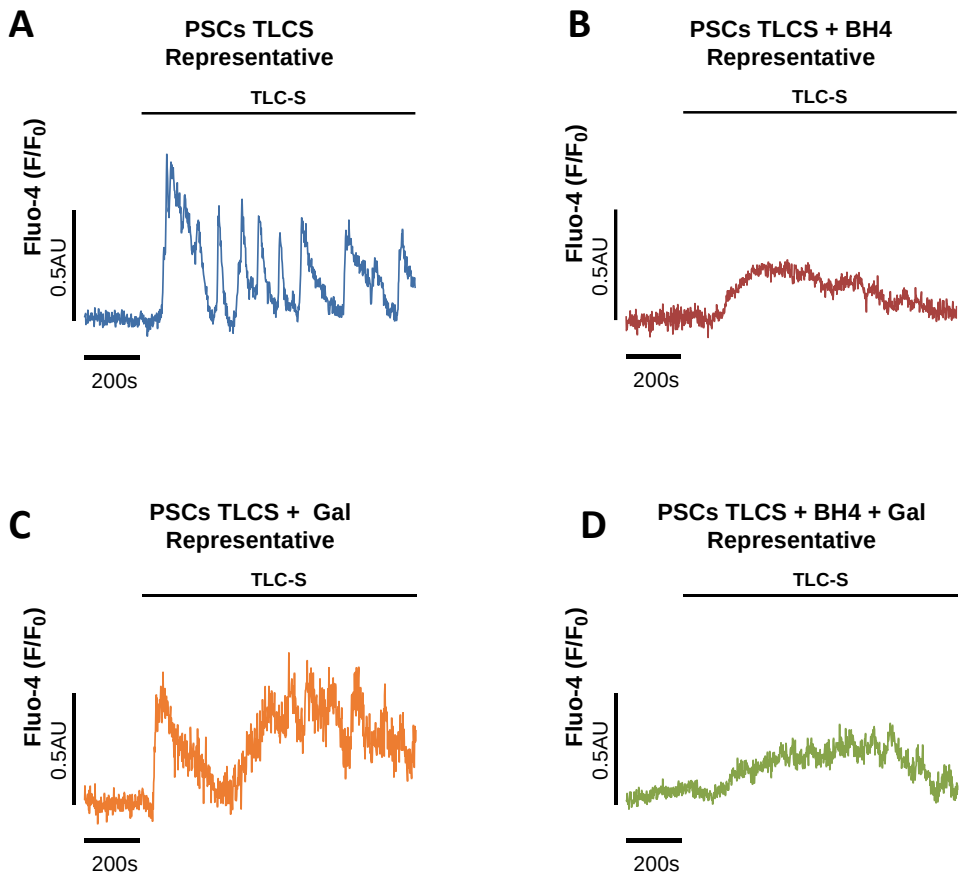



***Figure 3.6 Representative cytosolic calcium traces of PSCs in response to TLC-S stimulation.***

*Representative cytosolic calcium traces of PSCs in response to 400 µM TLC-S stimulation. Cells were either* ***(A)*** *untreated controls with DMSO addition (n=15),* ***(B)*** *treated with 50 µM BH4-Bcl2 peptide (n=11),* ***(C)*** *treated with 1 mM Galactose with no peptide present (n=14),* ***(D)*** *a combination treatment of 50 µM of a BH4-Bcl2 peptide and 1 mM Galactose (n=12). Cells were pre-incubated for 15mins with respective treatments and 400 µM TLC-S was applied at 200 seconds. Buffer in every experiment contained 2mg/10mls of trypsin and chymotrypsin inhibitor.*

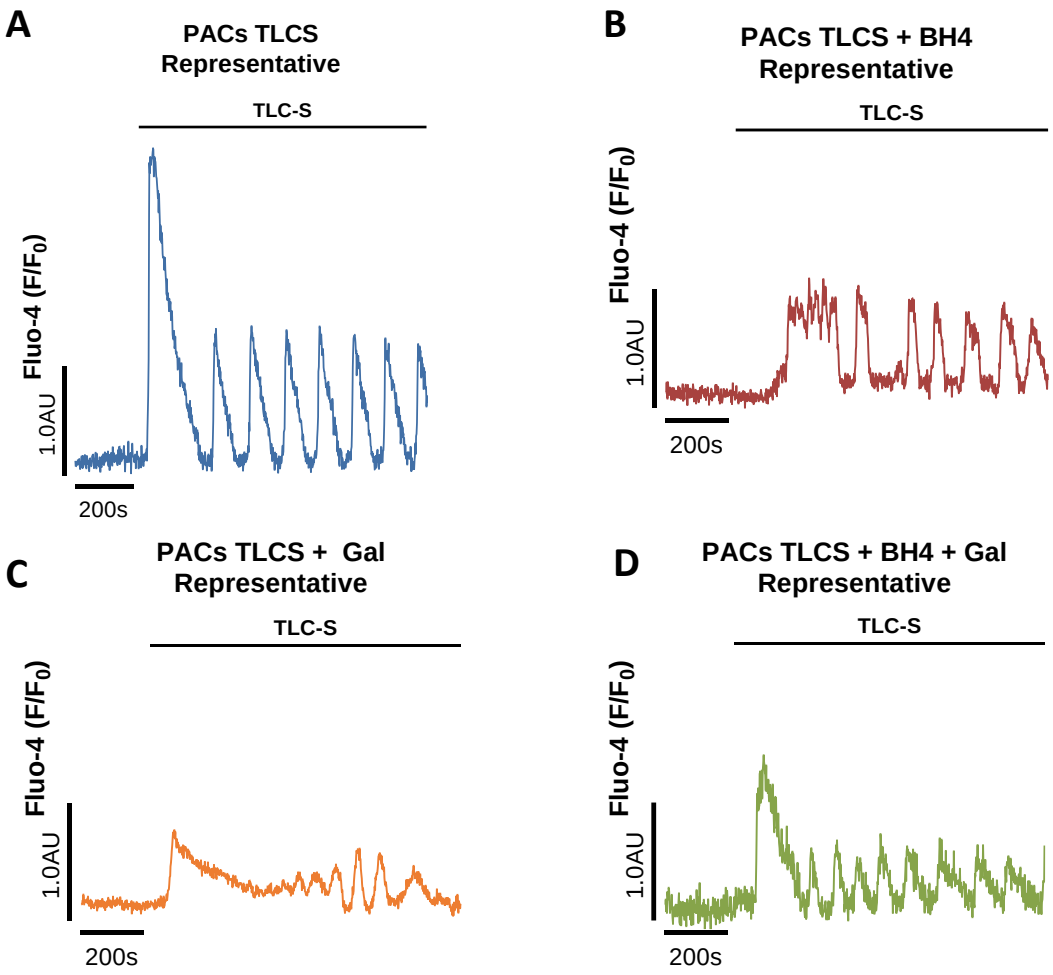



***Figure 3.7 Representative cytosolic calcium traces of PACs in response to TLC-S stimulation.***

*Representative cytosolic calcium traces of PACs in response to 400 µM TLC-S stimulation. Cells were either **(A)** untreated controls with DMSO addition (n=67), **(B)** treated with 50 µM BH4-Bcl2 peptide (n=20), **(C)** treated with 1 mM Galactose with no peptide present (n=22), **(D)** a combination treatment of 50 µM of a BH4-Bcl2 peptide and 1 mM Galactose (n=44). Cells were pre-incubated for 15mins with respective treatments and 400 µM TLC-S was applied at 200 seconds. Buffer in every experiment contained 2mg/10mls of trypsin and chymotrypsin inhibitor.*

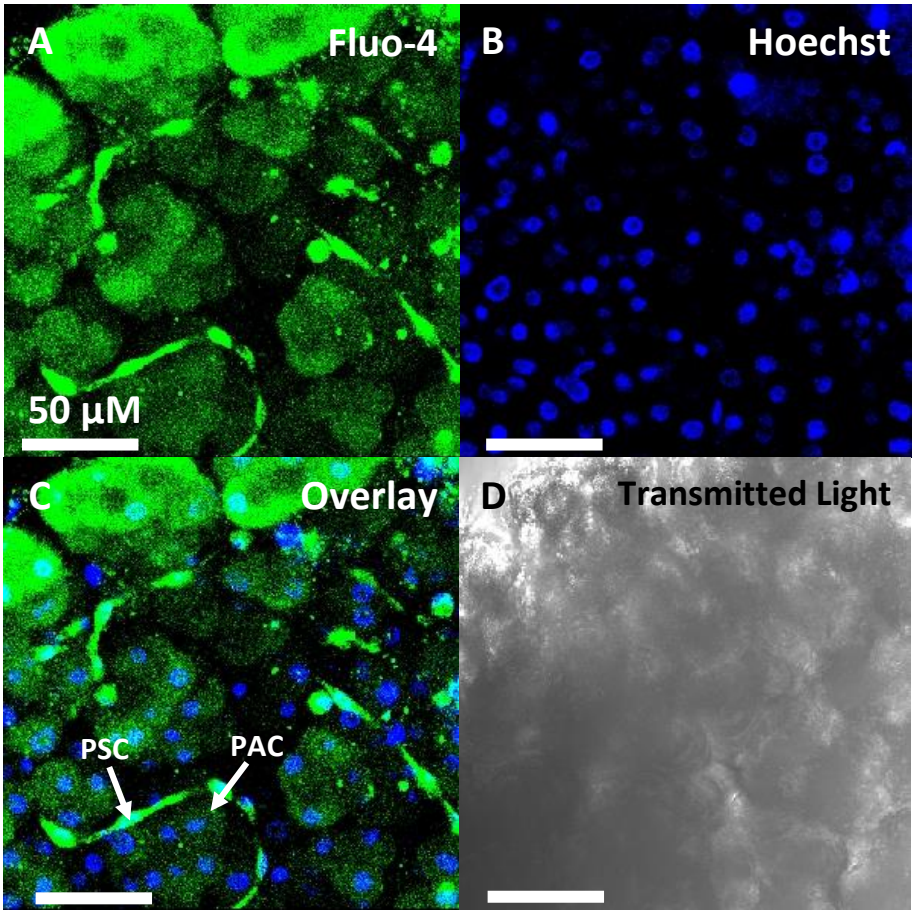



***Figure 3.8 Measurement of intracellular calcium in PACs and PSCs within Pancreatic Lobules.***

*Images obtained during the measurement of intracellular calcium levels in PSCs and PACs as indicated.* ***(A)*** *Image showing cells stained with Fluo-4-AM which highlights PSCs surrounded by PACs.* ***(B)*** *Demonstrating the Hoechst-stained nuclei within cells.* ***(C)*** *Overlaying image of Fluo-4-AM and Hoechst-stained cells within Pancreatic Lobules.* ***(D)*** *Visual light image of the Pancreatic Lobules under investigation.*

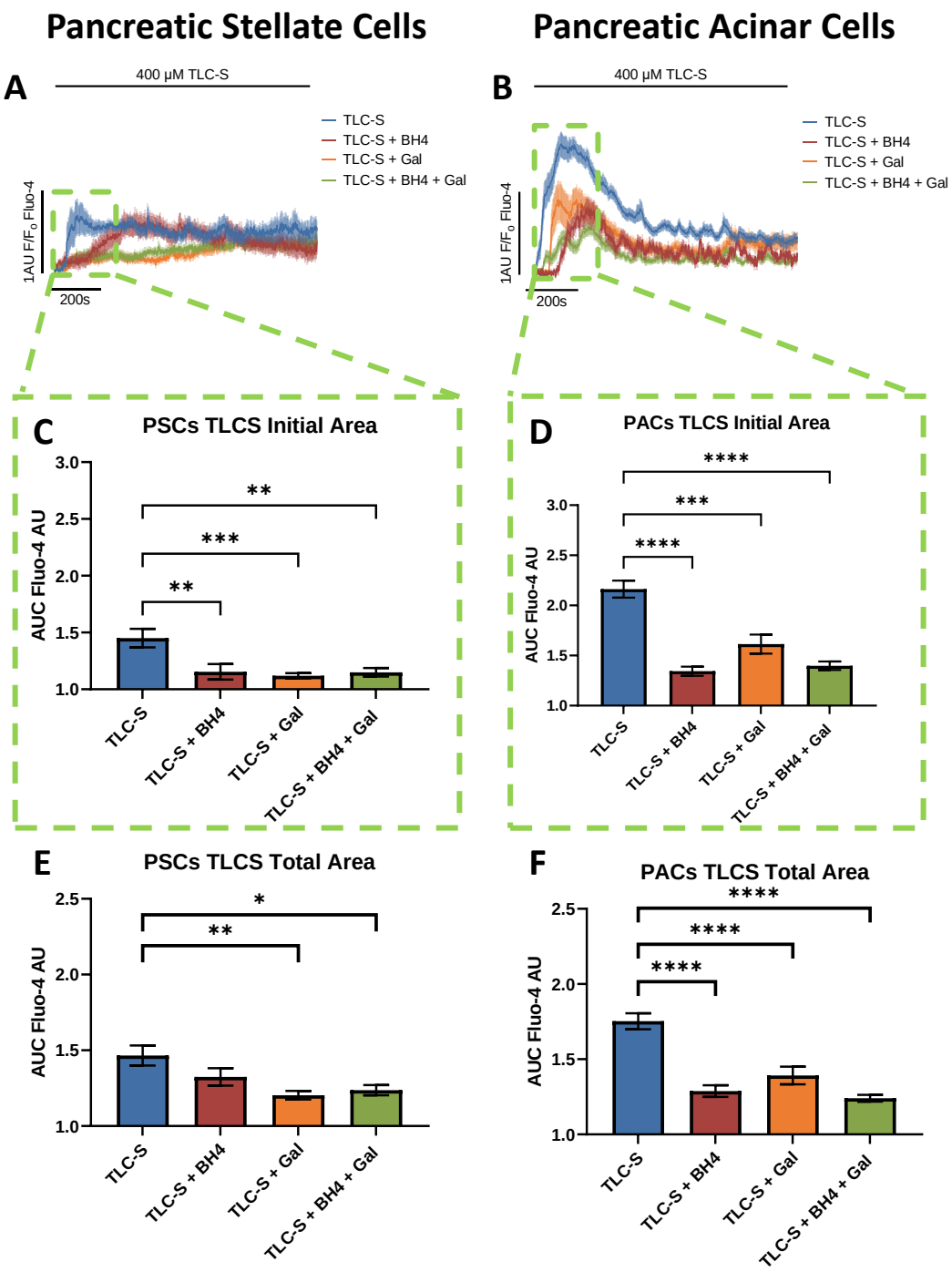
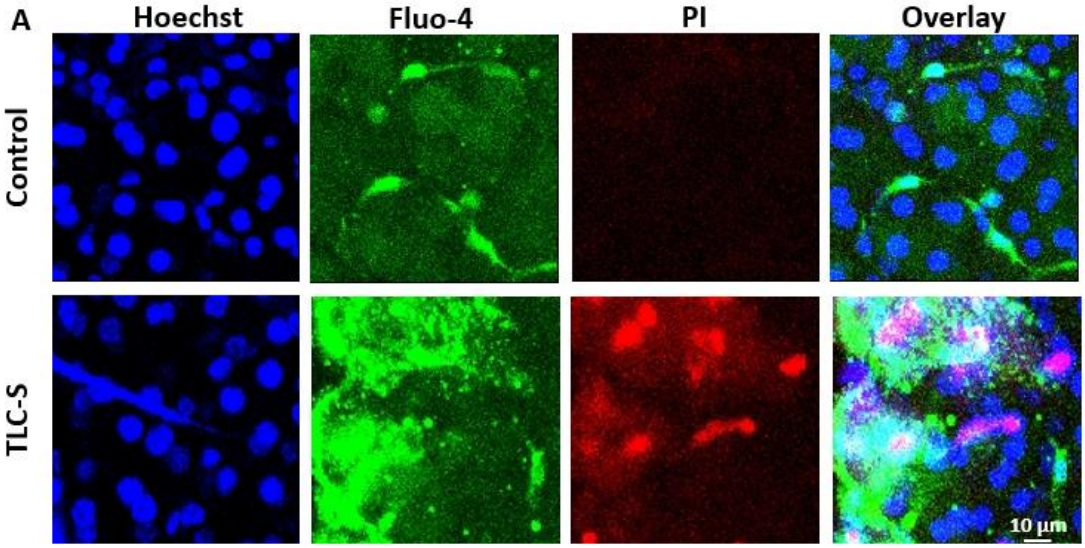

Pancreatic Stellate Cells
Pancreatic Acinar Cells
A
B
400 µM TLC-S
TLC-S
TLC-S + BH4
TLC-S + Gal
TLC-S + BH4 + Gal
1AU F/F0 Fluo-4
200s
C
PSCs TLCS Initial Area
D
PACs TLCS Initial Area
AUC Fluo-4 AU
E
PSCs TLCS Total Area
F
PACs TLCS Total Area

***Figure 3.9 Quantitative analysis of cytosolic calcium of PSCs and PACs in response to TLC-S stimulation in pancreatic lobules.***

*Average cytosolic calcium traces of PSCs and PACs in response to 400 µM TLC-S stimulation.* ***(A)*** *Shortened, quantified regions of average cytosolic calcium of PSC traces in response to 400 µM TLC-S stimulation in isolated pancreatic clusters were plotted with corresponding ±SEM values. Cells were either untreated controls with DMSO addition (n=15), treated with 50 µM BH4-Bcl-2 peptide (n=11), treated with 1 mM Galactose with no peptide present (n=14) or a combination treatment of 50 µM of a BH4-Bcl-2 peptide and 1 mM Galactose (n=12).* ***(B)*** *Shortened, quantified regions of average cytosolic calcium of PAC traces in response to 400 µM TLC-S stimulation in isolated pancreatic clusters were plotted with corresponding ±SEM values. Cells were either untreated controls with DMSO addition (n=67), treated with 50 µM BH4-Bcl2 peptide (n=20), treated with 1 mM Galactose with no peptide present (n=22) or a combination treatment of 50 µM of a BH4-Bcl2 peptide and 1 mM Galactose (n=44). Average traces were plotted with corresponding ±SEM values. Cells were pre-incubated for 15mins with respective treatments and 400 µM TLC-S was applied at 200 seconds following the start of recording. Buffer in every experiment contained 2 mg/10mls of trypsin and chymotrypsin inhibitor. The sets of traces in (A-B) represent the quantified areas analysed in (C-F). The entirety of the traces in (A-B) were analysed to determine total area of responses whilst the highlighted boxes indicate the region analysed to indicate difference within the initial areas of each trace. The average AUC.$s^{-1}$ values for the peak plateau area of responses were quantified in* ***(C)*** *for cytosolic traces in PSCs shown in (A) and* ***(D)*** *for cytosolic traces in PACs shown in (B). Average AUC.$s^{-1}$ values for this region was calculated from 200s to 450s from the start of recording and plotted with ±SEM. Similar statistical differences were observed in both PSCs and PACs. In PSCs, there was significant differences shown between 1mM Gal treatment and combination treatment of 50 µM BH4 and 1 mM Gal when compared to the control dataset. Both presented P-Values of <0.0001. Meanwhile, a statistical difference with a P-Value of 0.0365 was shown between the BH4 treatment and the control dataset. Furthermore, a statistical difference between 50 µM BH4 treatment and 1 mM Gal*

*treatment was highlighted with a P-Value of 0.0218. As for PACs, statistical difference was demonstrated between all treatment groups and the control with all P-Values equating to <0.0001 apart from 1mM Gal which was 0.0003. The average AUC.s$^{-1}$ values for the total area of responses were quantified in* ***(E)*** *for cytosolic traces in PSCs shown in (A) and* ***(F)*** *for cytosolic traces in PACs shown in (B). Total AUC.s$^{-1}$ values were calculated from 200s to 1050s from start of experiment and plotted with ±SEM. In PSCs significant differences were observed only between control treatments and 1 mM Gal along with the combination of 50 µM BH4 and 1 mM Gal with respective P-Values of 0.0017 and 0.0105. In PACs there were statistical differences observed between all treatment groups when compared to the control dataset, all with P-Values of <0.0001. (* = P-Value <0.05, ** = P-Value <0.01, *** = P-Value <0.001, **** = P-Value <0.0001).*


A
Hoechst
Fluo-4
PI
Overlay
Control
TLC-S
10 µm

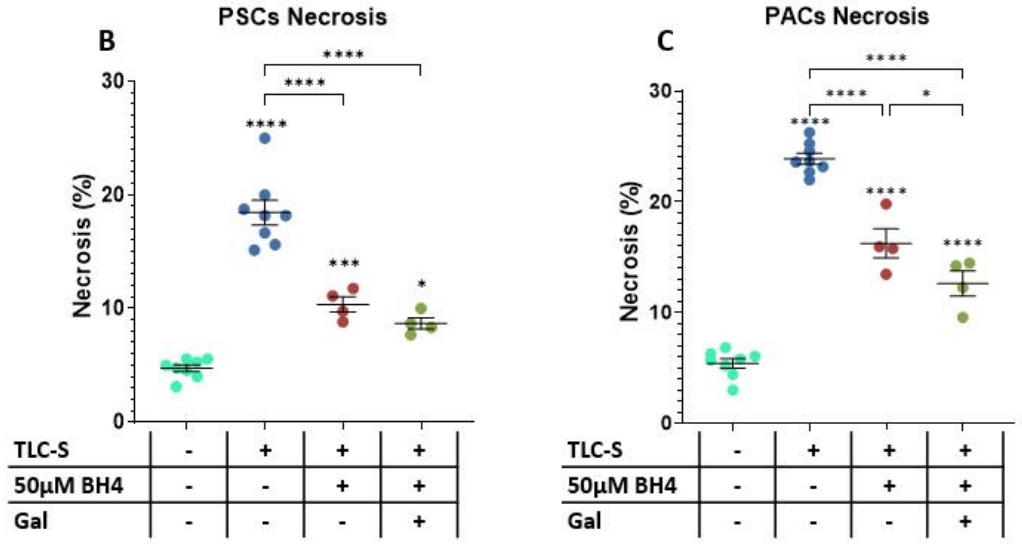

B
PSCs Necrosis
Necrosis (%)
TLC-S
50µM BH4
Gal
C
PACs Necrosis
Necrosis (%)
TLC-S
50µM BH4
Gal

***Figure 3.10 Necrotic cell death assay of PSCs and PACs in pancreatic lobules in response to 400 µM TLC-S.***

***(A)*** *Images obtained during assessing necrosis levels in PSCs and PACs when incubated with 400 µM TLC-S. The differing images highlight the structure of PSCs among PACs, the cells' nuclei as well as necrotic cells. Hoechst staining highlights the nuclei within cells. Intracellular calcium levels obtained with loading cells with Fluo-4-AM. This image also highlights the morphology of PSCs within clusters of PACs. Staining cells with propidium iodide highlights the necrotic cells within the cluster.* ***(B)*** *Quantification of necrosis assays of PSCs displaying BH4-Bcl-2 protection in response to 400 µM TLC-S. Pancreatic lobules were treated under negative control conditions (n=8), positive control conditions with 400 µM TLC-S (n=8), incubation with 50 µM BH4 solely (n=4) or with a combination of 50 µM BH4 and 1 mM Gal (n=4). Following pre-incubation of 15mins with treatments, pathological stimuli were applied to induce cell death over a 2-hour period, at which point necrosis was assessed. The percentages of propidium iodide-positive necrotic cells were assessed for each experimental condition. Significant differences were shown with a P-Value of <0.0001 between all treatment groups and the positive control. Whilst the treatment of 50 µM BH4-Bcl-2 yielded a p-value of 0.0009 when compared to the negative control. Furthermore, the combination treatment of 50 µM BH4-Bcl-2 and 1 mM Galactose produced a significant difference of 0.0207 compared to the negative control.* ***(C)*** *Quantification of necrosis assays of PACs response to 400 µM TLC-S. The necrosis of PACs was also determined within the same pancreatic lobules investigated in (A&B). Similar to the findings in PSCs, PACs displayed significant differences with a P-Value of <0.0001 was between all treatment groups and the positive control. However, an additional significant difference was displayed between the combination treatment of BH4 and Gal and the sole application of BH4 with a P-Value of 0.0364. All statistical differences directly above treatment groups represent comparison to negative control group (* = P-Value <0.05, ** = P-Value <0.01, *** = P-Value <0.001, **** = P-Value <0.0001).*

### 3.6 Discussion - Protective Effects of BH4-Bcl-2 in PACs and PSCs

The investigation of the Bcl-2 family of proteins over the past few decades has produced astonishing results ranging from its involvement in apoptosis to potential cancer treating properties (321–325,343). The multidomain carrying structure enables multiple different targets. Even more, this enables extremely specific targeting when these domains can be isolated. The synthesis of these domains has stimulated research into treating various pathologies including pancreatic pathology. The discovery and investigation of a novel BH4-Bcl-2 peptide showed extremely promising results regulating and providing protective effects vs cell death and pathological calcium signalling (322,326,329,330). Furthermore, its specificity for inhibition delivers distinct areas to target pathological signalling within pancreatic cells and acute pancreatitis (322,329).

A great deal of work investigating Bcl-2 peptides has been carried out by Vervliet *et al*. (329) and his team. Initially in previous studies, Vervliet *et al.* (326,330) demonstrated the preliminary inhibitory properties of Bcl-2 peptides including Bcl-$X_L$, Bcl-2 and the specific BH4 domain of these proteins. These early studies exhibited the inhibition of RyR activation by Bcl-2 peptides. However, these studies utilised RyR activation via pharmacological methods. As a consequence, further studies were carried out by Vervliet *et al*. (329) using the novel BH4-Bcl-2 peptide investigated RyR activated triggered by hormonal stimulation which is more characteristic of physiological conditions. Vervliet *et al*. (329) demonstrated significant reductions in cytosolic calcium signalling in response to CCK, therefore, signifying that NAADP/RyR mediated signalling is affected. Not only did Vervliet *et al*. (329) show the suppression of NAADP/RyR mediated signalling via the application of BH4 domains but showed the dampening of cytosolic calcium overload as a consequence. Additionally, Vervliet *et al*. (329) demonstrated that Pathological RyR-mediated cytosolic calcium overload was also drastically reduced upon application of BH4 domains. Therefore, indicating the potential for the application of BH4 domains as this cytosolic calcium overload is a key indicator of the development of Acute Pancreatitis. Moreover, this therapeutic potential was

also supported by the protective effects of BH4 domains against cell death in PACs during the application of necrosis inducing stimuli. The work carried out by Vervliet *et al*. (329) undoubtedly identified the potential therapeutic use of BH4-Bcl-2 in combating pathological calcium signalling and acute pancreatitis.

As a consequence of pathological signalling, the mitochondria of cells are significantly affected. Yet, the mitochondria are essential for cell survival. During physiological calcium signalling, calcium ions are transported into the mitochondria to aid ATP production (35). However, throughout pathological calcium signalling, the unwanted extreme influx of calcium into the mitochondria negatively affects the mitochondria's ability to produce ATP (116,117,261). This extreme influx of calcium into mitochondria disrupts the electrical potential of the mitochondrial membranes (261). These potentials are vital for the mitochondria to carry out its function to produce ATP for the cell. As a consequence, pathological calcium signalling prevents sufficient ATP production, therefore, acts as a contributing factor towards necrosis of PACs during pathological signalling. Due to the importance of mitochondria for cell survival, it was hypothesised to build upon the work carried out by Vervliet *et al*. (329) and to investigate the effects of BH4-Bcl-2 upon mitochondrial calcium signalling.

Mitochondria within PACs are not only vitally important for cell survival but are also critical to the functioning of the cell (35,149,258). The mitochondrial belt separating the two regions of the cell aid the creation of distinct calcium concentrations as well as functioning as a buffering tool to aid calcium homeostasis (140,344). Therefore, this study sought to investigate the ability of BH4-Bcl-2 domain peptide to regulate cytosolic and mitochondrial $Ca^{2+}$ signals produced by physiological and pathophysiological stimuli in PACs *in vitro* and in both PSCs and PACs *in situ*.

The initial findings of this project monitoring physiological signalling clearly demonstrated increased calcium levels within the cytosol and mitochondria shown in Fig. 3.2 and App. 1. This calcium signal within mitochondria is noticeably characterised by a sudden uptake of calcium into the mitochondria followed by a

sustained plateau which steadily declines towards the baseline. Consequently, the treatment of cells with the BH4-Bcl-2 peptide led to a significant reduction in mitochondrial calcium signalling. The mechanism of action of BH4-Bcl-2 is primarily the binding with $IP_3$ and Ryanodine receptors preventing the efflux of calcium from the endoplasmic reticulum. This is thought to have an impacting effect on the influx of calcium from the cytosol into the mitochondria. Consequently, reducing the calcium response seen within mitochondria. Furthermore, during mitochondrial calcium responses, there is additional calcium movement from the endoplasmic reticulum directly into the mitochondrial intermembrane space via the mitochondrial-associated ER membrane which consist of $IP_3$ receptors and voltage-dependent anion channels (VDAC). Potential further binding to these $IP_3$ receptors may also compound the inhibitory effects of BH4-Bcl-2 upon mitochondrial calcium responses.

During responses to physiological concentrations of CCK within PACs in Fig. 3.2, the size and strength of the mitochondrial calcium response was reduced. Furthermore, this was also seen during responses to pathophysiological stimuli demonstrated in Fig. 3.3. Initially, these findings align with the results found by Vervliet *et al*. (329) confirming that the novel BH4-Bcl-2 domain reduces NAADP/RyR mediated signalling, most likely due to binding with RyRs, but is not limited to just RyRs. Therefore, further testing was undertaken to assess the effectiveness of BH4-Bcl-2 at reducing mitochondrial calcium overload.

The BH4-Bcl-2 peptide significantly reduced mitochondrial calcium levels in PACs during physiological and pathophysiological signalling. However, it did not completely inhibit calcium influx into the mitochondria. Therefore, it was theorised to treat cells with a combination of the BH4-Bcl-2 peptide alongside the sugar galactose. Galactose demonstrated extremely promising effects in previous work carried out by Peng *et al.* (276). It was hoped that this addition in combination with BH4 would show improved or total inhibition of the mitochondrial calcium influx. As it is much more important to prevent unwanted pathological calcium overload, it was deemed to test this using pathological stimuli. The combination of BH4 and

galactose did prove to be more effective than the sole application of BH4 and galactose as shown in Fig. 3.3. However, the addition of galactose alongside BH4 did not fully negate the effects of the pathological stimuli within the timeframe of the experiment. Although the mitochondrial calcium signal was not completely abolished, the addition of galactose with BH4 significantly affected the rate of the response. The combination of BH4 and galactose provided an extreme delay in response as quantified in Fig. 3.4. This delay may prove to be pivotal step in protecting cells against calcium overload by delivering more time for the mitochondria and cytosol to absorb and negate the increased calcium release/influx. The more time given to the cell to expel/store unwanted released calcium ions which may be beneficial in the fight against cell death and pathology.

Expanding on this idea, necrosis assays in Fig 3.5 were carried out on isolated PACs to test whether the addition of differing BH4 concentrations and combinations with galactose yielded any protective effects. Previous assays by Vervliet *et al*. (329) resulted in significant protection against necrosis which was granted by the addition of BH4 peptides. This study expanded upon this result and tested a lower concentration of BH4 in addition to a combinatory treatment with 1 mM galactose. This combination treatment yielded some promising results. The combination of a lower concentration of BH4 along with galactose produced similar levels of necrosis as compared to the sole application of a high concentration of BH4. This is extremely important and increases the likelihood of the potential use of lowering concentrations of BH4 in pathophysiological systems.

In light of these results, PACs are not the only cells that are affected during the development of Acute Pancreatitis. PSCs are also adversely affected during pathophysiological stress and contribute to the development of Acute Pancreatitis. As a consequence, the study's attention moved to investigate any protective effects of BH4 and galactose upon PSCs. Unfortunately, the study aimed to examine pathological mitochondrial calcium within PSCs, however, this was not possible due to the insufficient loading of Rhod-2-AM in PSCs. Therefore, cytosolic calcium was monitored in its place as shown in Fig. 3.6-9. These novel experiments

demonstrated some positive results in PSCs however, the protective effects of BH4 were not as great as those shown in isolated PACs. This may have been due to the density of the surround tissue as PSCs must be isolated within pancreatic lobules. Moreover, due to the size of these lobules, a greater concentration of TLC-S was required to produce a reliable response, therefore, the protective effects on PACs in pancreatic lobules was less than that seen within isolated PACs. The necrosis assays that were carried out did provide some reassuring results which were more similar to those seen in isolated PACs. The treatment of BH4 provided significant protection against necrosis in both PSCs and PACs however, the further addition of galactose provided even greater protection in PACs.

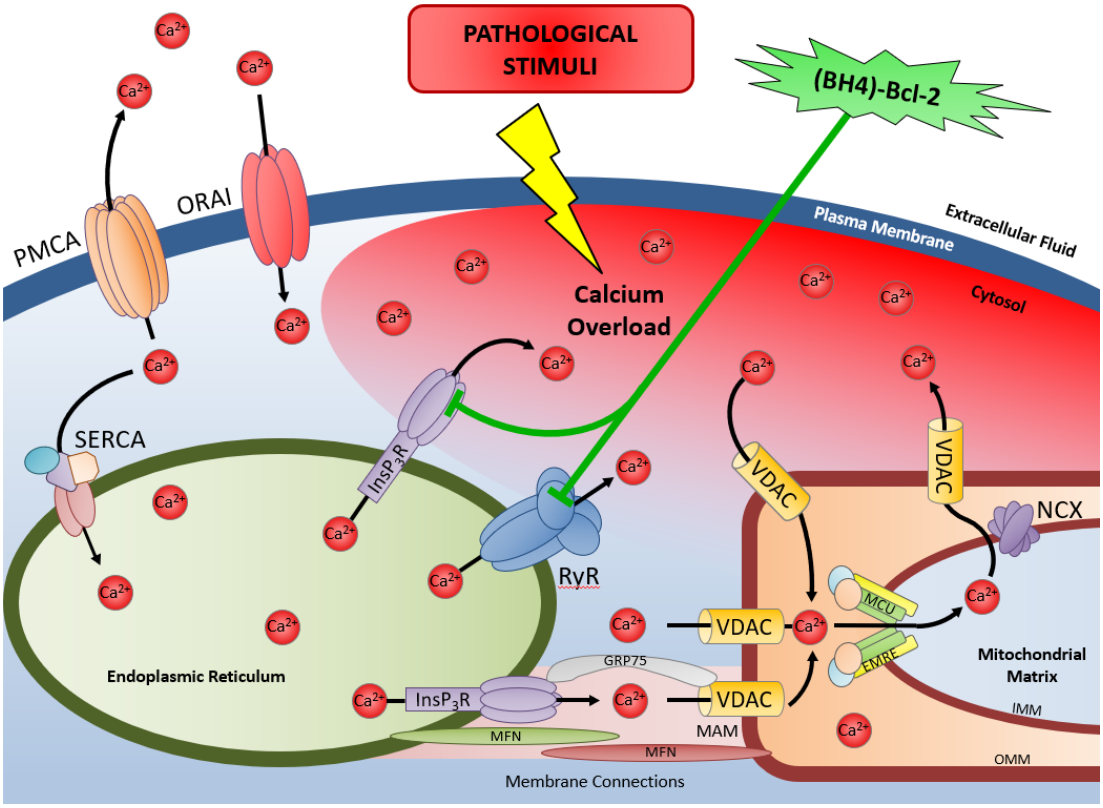



***Figure 3.11 Schematic summary representing the internal relationships between multiple $Ca^{2+}$ signalling mechanisms which are responsible for the initiation of calcium overload and pathology.***

*The application of a pathological stimuli induces calcium overload within the cytosol of the cell. This overload is caused due to the release of calcium from the ER via $IP_3$ and Ryanodine receptors which also induce additional calcium entry through ORAI channels. Furthermore, this cytosolic calcium overload increases the flow of calcium ions into the mitochondrial matrix via VDAC and MCU channels which disturbs the membrane potential of the mitochondrial membrane which is essential for mitochondrial ATP production.*

# Chapter 4 - Results

# Promising Protective Effects of BH4 Against Asparaginase-Induced Pathology

## 4.1 Introduction

Asparaginase is a known trigger of acute pancreatitis in patients being treated for Acute Lymphoblastic Leukaemia, which as previously mentioned, is the most common type of childhood leukaemia (312). The asparaginase utilised during the treatment of ALL is a common and effective way to treat lymphoproliferative diseases in children with an extremely high survival rate of nearly 90% in childhood ALL (314,317). However, a common side effect which occurs in approximately 5-10% of ALL patients who undergo asparaginase treatment develop acute pancreatitis. This occurrence of acute pancreatitis is severe enough to cease asparaginase treatment. Upon doing so, the ALL becomes untreated which significantly increases it severity (34).

This connection between asparaginase treatment and the development of acute pancreatitis had not been investigated until relatively recently by Peng *et al*. (34). During this study, a strong association between asparaginase and the development of acute pancreatitis was made. The application of asparaginase clearly demonstrated pathological effects within PACs. Asparaginase elevated intracellular calcium concentrations which also significantly reduced cellular ATP levels. These are well-known side effects and key elements of pathology within PACs. The study also demonstrated an association of asparaginase-induced pathology and PAR2 which was also linked with $IP_3$ and NAADP signalling as discussed in previous chapters. Finally, Peng *et al*. (34) showed the induction of cellular necrosis in response to asparaginase. Furthermore, Peng *et al*. (34) reduced the levels of necrosis by the addition calcium signalling inhibitors such as GSK-7975A. Not only were the levels of necrosis reduced but the pathology induced by asparaginase did not rely upon asparagine. As a consequence, any attempt to block/rescue these necrotic effects should not interfere with asparaginase's mode of action against ALL. Interestingly, further experiments carried out shown in *Peng et al.* (276) demonstrate further protection against asparaginase-induced necrosis whilst applying the sugar galactose(276). Therefore, demonstrating that these

pathological signals may be prevented by targeting calcium signalling and metabolic mechanisms (34).

Following the ground-breaking results of *Peng et al.* (34), preliminary experiments were carried out to assess the effects of asparaginase application upon PSCs. Furthermore, due to the isolation of PSCs in lobules, PACs are inherently isolated as well. As a consequence, measurements can be recorded in PACs in addition to PSCs. Shown in Fig. 4.1, the effects of asparaginase are highlighted in both PSCs and PACs. Compared to control levels, the maximum amplitude of the intracellular calcium response is significantly higher than the control values, indicating that asparaginase also exerts pathological effects upon PSCs. This provided a good founding to suggest the investigation of potential inhibitors/moderators, specifically the BH4-Bcl-2 peptide, of these induced calcium signals. As these signals are most likely pathological, it is inevitable that necrosis will ensue following this excessive calcium overload. Therefore, it was hoped that the BH4-Bcl-2 peptide may also provide beneficial protection against asparaginase induced pathology.

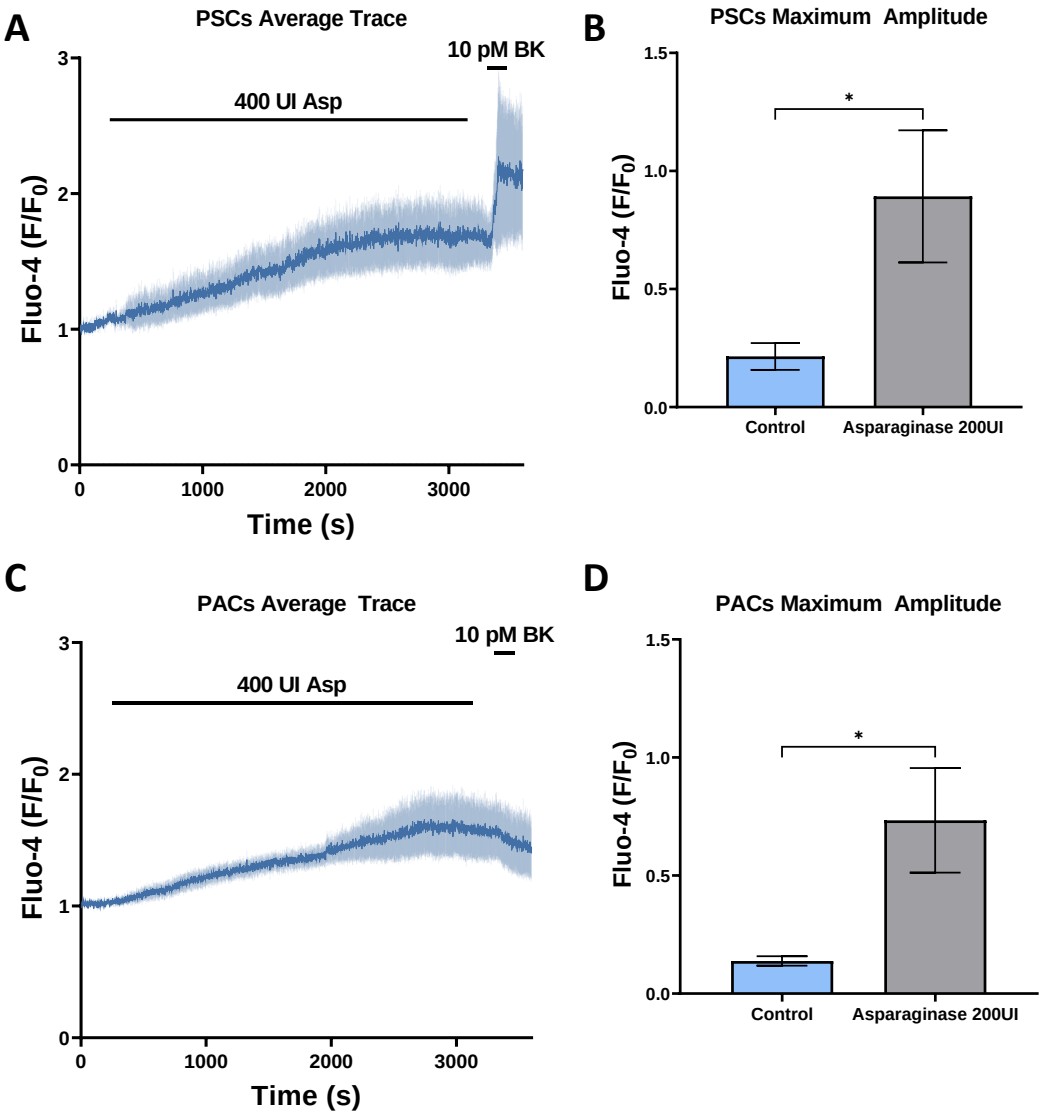

A
PSCs Average Trace
10 pM BK
400 UI Asp
Fluo-4 (F/F0)
Time (s)
B
PSCs Maximum Amplitude
Fluo-4 (F/F0)
Control
Asparaginase 200UI
C
PACs Average Trace
10 pM BK
400 UI Asp
Fluo-4 (F/F0)
Time (s)
D
PACs Maximum Amplitude
Fluo-4 (F/F0)
Control
Asparaginase 200UI

***Figure 4.1 Application of Asparaginase to PACs and PSCs within pancreatic lobules.***

*Average cytosolic calcium traces and analysis of pancreatic lobules loaded with Fluo-4-AM in response to 400 UI Asparaginase.* ***(A)*** *Average cytosolic traces of PSCs during the application of 400 UI of Asparaginase.* ***(B)*** *Quantified data based on traces shown in (A) highlighting a significant difference, with a p-value of 0.0449, asparaginase has upon the maximum cytosolic amplitude measured within PSCs. Control was taken as the initial baseline phase of the trace.* ***(C)*** *Average cytosolic traces of PACs during the application of 400 UI of Asparaginase.* ***(D)*** *Quantified data based on traces shown in (C) highlighting a significant difference, with a p-value of 0.0201, asparaginase has upon the maximum cytosolic amplitude measured within PSCs. Control was taken as the initial baseline phase of the trace. Modified figures from David L. Evans Master's Degree Project 2017.*

## 4.2 Results - Protective Effects of BH4-Bcl-2 Peptide During Asparaginase-Induced Pathology in PACs

Similarly, to section 3.4, in order to test the effectiveness of the BH4-Bcl-2 peptide against pathology, necrosis assays were carried out. In contrast to those experiments in 3.4, the pathological stimuli used here was asparaginase. Asparaginase is a known pathological stimuli and inducer of cell death. Similar concentrations and combinations of BH4-Bcl-2 were used including a high concentration (50 µM) of BH4-Bcl-2 peptide as well as a lower concentration (20 µM) of BH4-Bcl-2 peptide. Furthermore, combinatory treatments of both these concentrations were also used along with the addition of 1 mM of galactose. Overall, any application of the BH4-Bcl-2 peptide reduced cell death compared to the control mean of 5.576% ±0.2876 shown in Fig. 4.2.

However, the application of the lower 20 µM concentration of BH4-Bcl-2 yielded a lesser significant reduction of cell death compared to the higher 50 µM concentration of BH4-Bcl-2. 20 µM BH4 reduced cell death to an average 13.92% ±1.099 and provided a p-value of 0.0331 when compared to the positive control of 400 UI Asp with a mean of 17.45 ±0.7643. Whereas the 50 µM BH4 treatment reduced cell death to an average of 9.921% ±1.326 and produced a p-value of <0.0001 when compared to 400 UI Asp. In keeping with the experiments shown in Fig. 3.5, 1 mM Galactose was added in combination with the two difference concentrations of BH4. The results from these combinations again produced similar findings. The combination of 50 µM BH4 and 1 mM Gal yielded a mean cell death of 9.067% ±0.5517. When this was compared to 400 UI Asp, a p-value of <0.0001 was obtained whilst there was no statistical significance shown between this and the other combinatory treatment which produced a mean cell death of 10.48% ±0.7176. The combination of 20 µM BH4 and 1 mM Gal also produced a p-value of <0.0001 when compared to 400 UI Asp. Although, the results show that the combinatory use of a high dose of BH4-Bcl-2 and 1 mM galactose did not provide any further protection compared to solely applying 50 µM of BH4-Bcl-2 it does provide some reassuring findings. These results suggest that similar levels of protection against

cell death can be obtained with a lowered concentration of BH4 whilst also reducing the volume of DMSO addition is required. This reduction of DMSO will aid the viability to apply the BH4-Bcl-2 peptide as a therapeutic alternative.

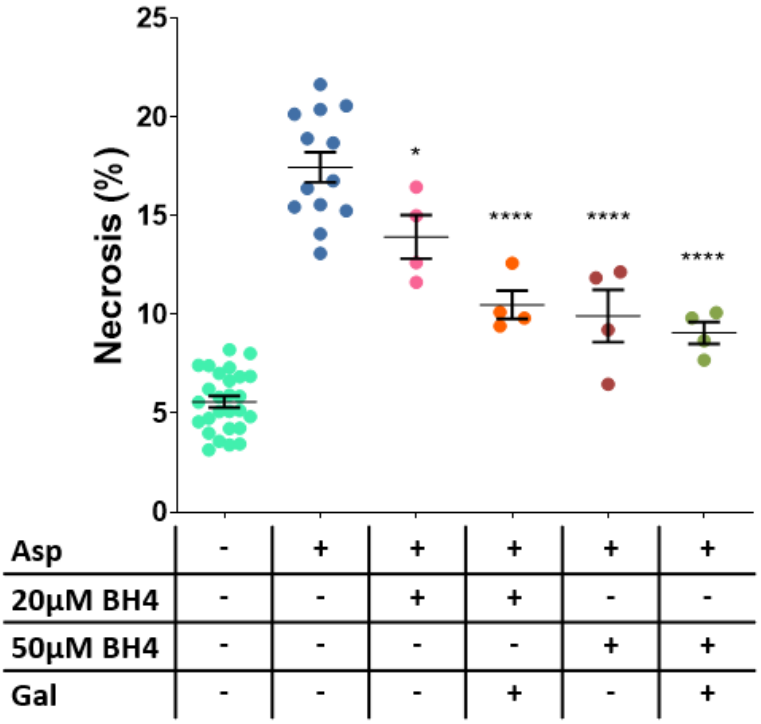



***Figure 4.2 Quantification of necrosis assays of isolated PACs displaying BH4-Bcl2 protection in response to asparaginase.***

*Isolated PACS were treated under numerous different conditions. Cells were either incubated under control conditions (n=27), with 200 UI Asparaginase (n=13), with 20 µM BH4-Bcl-2 (BH4) (n=4), with 20 µM BH4 and 1 mM Galactose (Gal) (n=4), with 50 µM BH4 (n=4), or with 50 µM BH4 and 1 mM Gal (n=4). Following pre-incubation of 15 mins with treatments, pathological stimuli were applied to induce cell death to every experiment, excluding the negative control, over a 2-hour period at which point necrosis was assessed after Nuclear Green DCS1 staining. The percentages of Nuclear Green DCS1-positive necrotic cells were assessed for each experimental condition. 200 UI Asparaginase was used to induce necrosis. Each data point represents an independent repeat of the experiment, with the average values ±SEM displayed (* = P-Value <0.05, ** = P-Value <0.01, *** = P-Value <0.001, **** = P-Value <0.0001).*

### 4.3 Discussion

The findings of these results clearly show the ability to use the BH4-Bcl-2 to reduce necrosis and protect cells from cell death afflicted by asparaginase. Not only that, but these results also highlight and confirm the findings from Chapter 3, suggesting the combination of applying BH4-Bcl-2 alongside galactose provides the strongest protection against pathological stimuli including asparaginase.

Initially this Chapter was aimed to investigate the protective effects of BH4-Bcl-2 against multiple scenarios which include asparaginase as a pathological agent. It was planned for this to involve cytosolic and mitochondrial signalling in response to asparaginase in both pancreatic acinar and stellate cells. Essentially the experiments seen in Chapter 3 were to be repeated whilst substituting the TLC-S for asparaginase. However, due to the suspension of laboratory experiments due to COVID-19 this was made impossible. Additionally further time constraints made the continuation of these experiments impractical in order to complete this study by the specified deadline.

# Chapter 5 - Results

# Potential Therapeutics of Acute Pancreatitis Targeting Calcium Entry Channels

## 5.1 Introduction

Previous experiments using these techniques have highlighted the significant role of calcium overload in stellate cells during both alcohol and bile-induced acute pancreatitis (172,279). As both cell types (PACs and PSCs) have been shown to be involved in the development of acute pancreatitis via multiple mechanisms, highlighted in Fig. 1, there is a necessity to develop inhibitory compounds which suppress excessive calcium release and calcium entry into the cell. However, due to the previous findings in stellate cells showing an involvement in the development of acute pancreatitis, these inhibitory compounds should control calcium overload in both PACs and PSCs in attempting to combat acute pancreatitis.

Some studies have demonstrated numerous times that calcium signalling within PACs can be effectively inhibited. As previously eluded to, the work carried out by Gryshchenko *et al.* (272) demonstrated the unique signalling responses in PSCs compared to PACs as shown in Fig. 5.1A. The inability to evoke BK-elicited calcium responses in PACs clearly shows the difference in signalling mechanisms and receptors. More interestingly, as shown in Fig. 5.1B, Gryshchenko *et al.* (272) demonstrated part inhibition of the BK-elicited response in PSCs. Highlighting the reliance BK-mediated signalling has upon calcium entry. By utilising the CRAC channel inhibitor, GSK-7975A, Gryshchenko *et al.* (272) blocked the plateau phase of the calcium response. As SOCE is a key element of the amplification of pathological calcium signalling, it provides an interesting avenue to investigate in the search for potential therapeutic tools against acute pancreatitis.

Preliminary experiments that were carried out have indicated possible inhibition of BK signalling in PSCs with another compound, NED-19 as shown in Fig 5.2. However, the entire bradykinin-induced signal was not blocked. As shown in Fig. 5.2, the initial phase of the signal was not affected. This phase is predominantly due to the release of calcium from intracellular stores (37,38,172,189,222,272,331). Whereas the second plateau-like phase of the signal is caused by calcium entry. This second phase was completely abolished by the application of NED-19 therefore indicating that NED-19 may inhibit SOCE signalling and CRAC. NED-19 has been well

documented in inhibiting NAADP-linked calcium signalling in PACs, therefore, potentially suggesting NED-19 as a good inhibitor to investigate further.

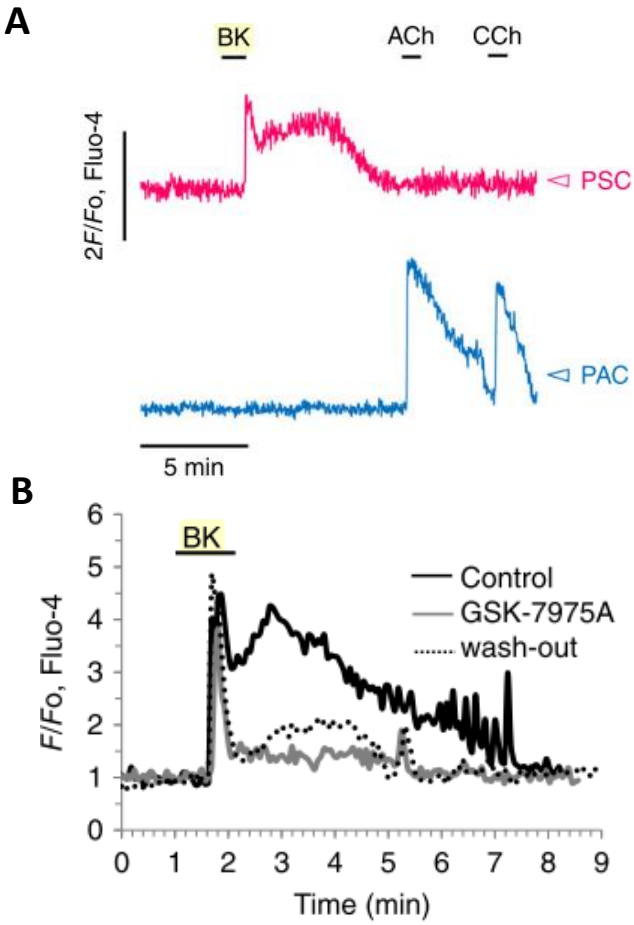
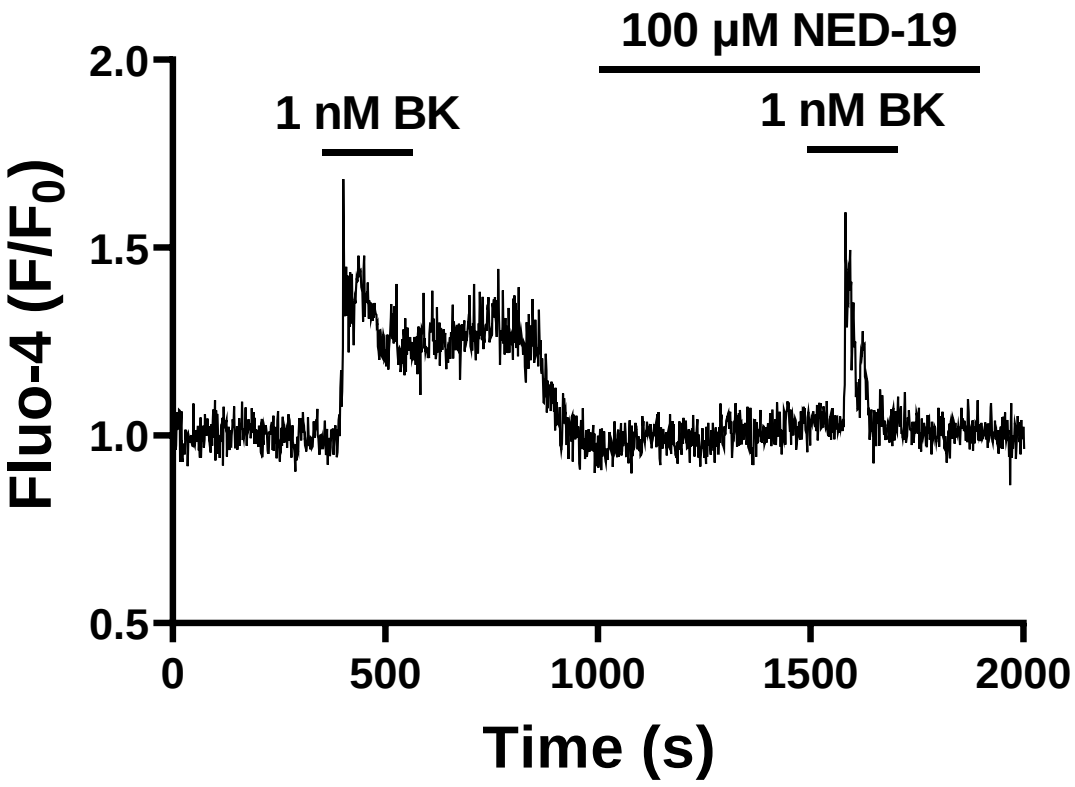



***Figure 5.1 Seperated mechanisms of $Ca^{2+}$ signal generation in PSCs and neighbouring PACs***

***(A)*** *Intracellular $Ca^{2+}$ measurements recorded in PSCs and neighbouring PACs within a pancreatic lobule demonstrate the difference in signal generation between cell types when BK, ACh and CCh were applied. Specifically, PSCs responded to 1 nM application of BK whereas PACs demonstrated no response to BK. Furthermore, PACs responded to 10 µM application of ACh and CCh whereas PSCs did not.* ***(B)*** *The application of the CRAC channel inhibitor GSK-7975A (10 µM) demonstrated a reduced plateau phase of BK-elicited signalling. Additionally, this inhibited signal was partially restored following washing out of the inhibitor. Results were produced from Gryshchenko et al. (272).*

**A**


100 µM NED-19
1 nM BK
1 nM BK
Fluo-4 (F/F0)
2.0
1.5
1.0
0.5
0
500
1000
1500
2000
Time (s)

**B**

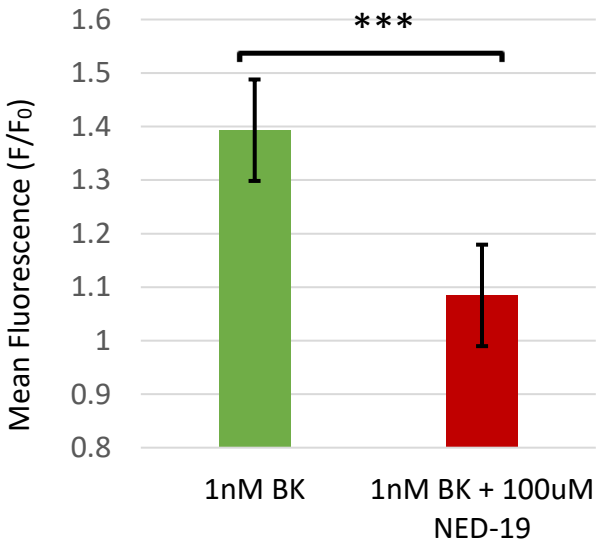

***
Mean Fluorescence (F/F0)
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
1nM BK
1nM BK + 100uM NED-19

***Figure 5.2 Intracellular calcium levels induced by bradykinin stimulation and during NED-19 application in PSCs.***

***(A)*** *Representative trace monitoring intracellular $Ca^{2+}$ levels within PSCs during 1 nM Bradykinin (BK) stimulation and 100µM NED-19 application. Additionally, the cells were incubated for 13mins with NED-19 during the recording.* ***(B)*** *Mean intracellular $Ca^{2+}$ concentrations during the 1 nM BK stimulation and 1 nM BK stimulation during 100µM NED-19 application (n = 3, *** = P<0.001). Figures modified from David L. Evans Master's Degree Project 2017.*

The overload of cytosolic calcium is a well-known hallmark of acute pancreatitis as previously described. The premature activation of digestive enzymes and proenzymes within the cell is caused by prolonged elevation of global cytosolic calcium levels. As stated, this increased calcium level is due to calcium released from intracellular calcium stores which is followed by excessive $Ca^{2+}$ entry through SOCE channels such as CRAC channels. The drive of calcium into the cytosol via the complex number of previously mentioned mechanisms open several different avenues of intervention which could prevent pathology. These span from inhibition of agonist binding, CICR or calcium entry.

Lately, as CRAC channels are the prime mediator of SOCE channels in PACs, they have become a key target in the perseverance to prevent pathology. These CRAC channels are constructed from two main sections. Firstly, a pore forming protein named Orai1 and secondly a $Ca^{2+}$ sensing protein commonly known as STIM-1. Again, as previously discussed, upon the depletion of calcium from intracellular stores, STIM-1 activates and opens the Orai1 channel in the cellular membrane. This opening allows $Ca^{2+}$ to flow directly into the cytosol of the cell, down its electrochemical gradient. As a consequence, specific molecules/drugs have been created in order to try and prevent this influx of calcium via CRAC channels. An example of this is a compound produced by CalciMedica, CM4620 (35,170,297). This compound has been investigated in animal and cell models on numerous occasions. This CRAC channel inhibitor has demonstrated some promising results inhibiting pathology and unwanted pathological calcium signalling. As a consequence, CM4620 is currently being investigated in ongoing clinical human trials (341).

## 5.2 Results - Utilising Calcium Entry Inhibitors as Potential Therapeutics for Acute Pancreatitis

The use of ACh as a stimulant to initiate intracellular calcium signalling has been known for decades and is clearly confirmed in Fig. 5.3a. Here, clear oscillatory spikes of cytosolic calcium are produced when physiological concentrations of 20 nM ACh are applied to PACs. Furthermore, the application of 100 nM of the CRAC channel inhibitor CM4620 shows reduced oscillatory signalling. Visually comparing the representative traces in Fig. 5.3 and the average traces in Fig. 5.4, 100 nM CM4620 clearly reduces the frequency of cytosolic spiking in addition to reducing the length of signal induced when a high concentration of 1 µM ACh was applied. These visual differences were also confirmed during quantitative comparisons between the two datasets in Fig. 5.5 and Fig. 5.6. The traces were analysed in two distinct regions as well as two different parameters. Firstly, the AUC and maximal amplitudes for each trace were calculated during the physiological signalling period where 20 nM ACh was applied. These results, shown in Fig. 5.5 highlight significant differences between the application of 100 nM CM4620 and the control dataset. CM4620 reduced the maximum amplitude from a control mean of 2.886 ±0.1754 to an average of 2.298 ±0.1267 and produced a p-value of 0.0301. Furthermore, it significantly reduced the total AUC from a control mean of 1.774 ±0.0991 to an average of 1.476 ±0.0366 whilst yielding an even greater statistical p-value of 0.0016.

However, the analysis of the response to 1 µM ACh provided differing results from those seen during 20 nM ACh application. Comparison of the AUC, 100 nM CM4620 significantly reduced the area of response with a p-value of <0.0001 and reducing the mean area to 1.233 ±0.0533 from a control mean of 1.780 ±0.1285. This difference was not seen when comparing the maximum amplitude during the 1 µM ACh application. There was no significant difference between the control mean of 2.634 ±0.2121 and the mean of CM4620 of 2.419 ±0.1693 with an observed p-value of 0.5344. When visually comparing the average traces, it's clear that the recovery phase of the 1 µM ACh evoked response was affected by 100 nM CM4620.

Furthermore, other extensive studies investigated this CRAC channel inhibitor, CM4620 in more depth and showed reliable inhibition of calcium signals induced by multiple pathological agents. It was also shown that CM4620 provided strong protective effects against necrosis, again induced by pathological agents. All of these results suggests that CM4620 should be considered as a strong therapeutic for acute pancreatitis. Furthermore, the results also confirm that the importance of CRAC channels in the induction of calcium signals and so, the development of acute pancreatitis.

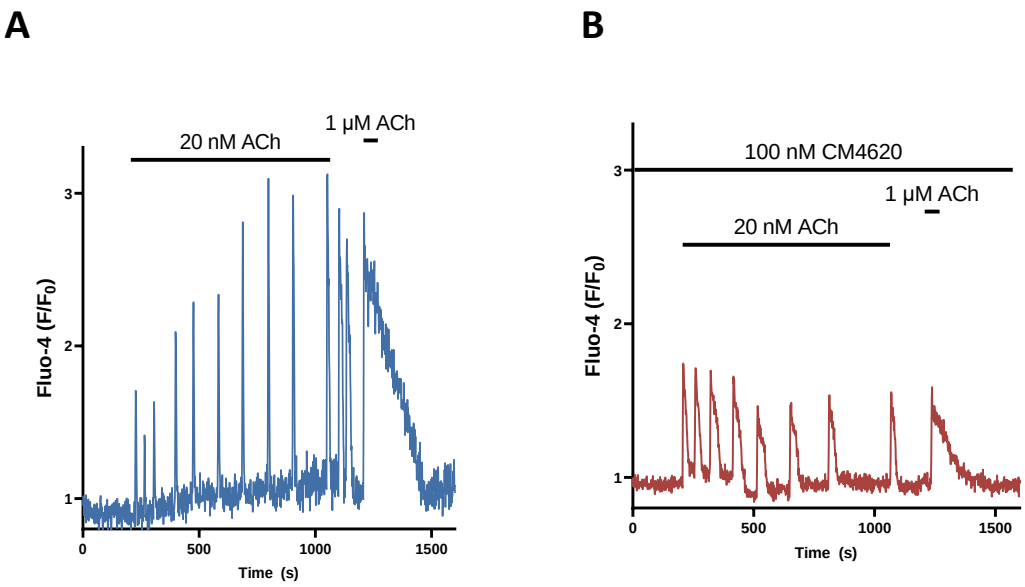



***Figure 5.3 Representative calcium traces of control and CM4620 treated pancreatic acinar cells following ACh application.***

*Cells were exposed to ACh which stimulated oscillatory calcium signalling within PACs. Cells were treated with either **(A)** a control $Na^+$ HEPES buffer (n=7) or **(B)** a cell treatment containing 100 nM of the CRAC channel inhibitor CM4620 (n=25).*

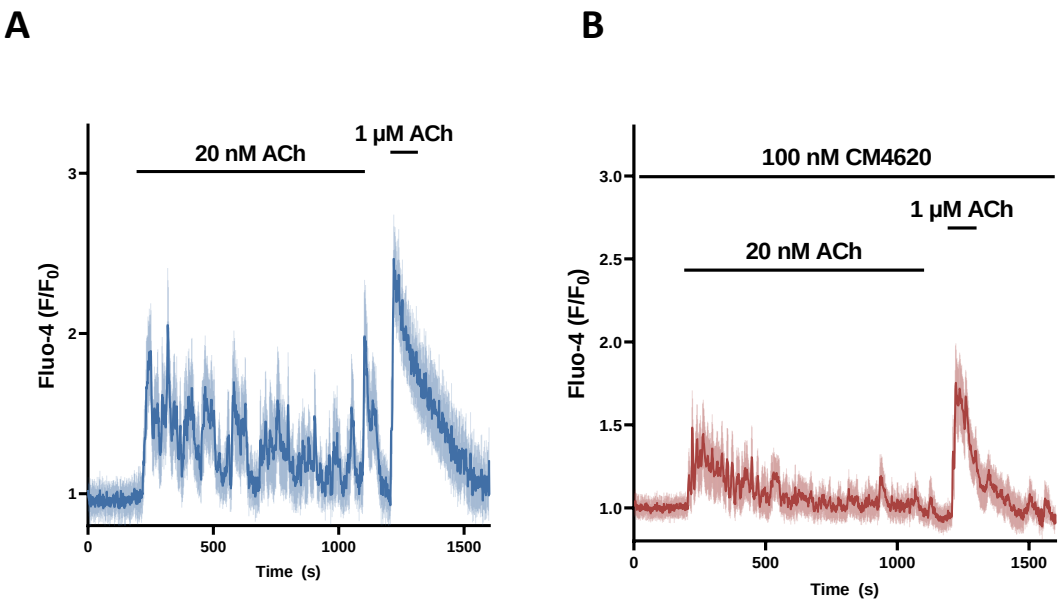



***Figure 5.4 Average calcium traces of control and CM4620 treated PACs following ACh application.***

*Cells were exposed to ACh which stimulated oscillatory calcium signalling within PACs. Cells were treated with either* ***(A)*** *a control $Na^+$ HEPES buffer (n=7) or* ***(B)*** *a cell treatment containing 100 nM of the CRAC channel inhibitor CM4620 (n=25).*

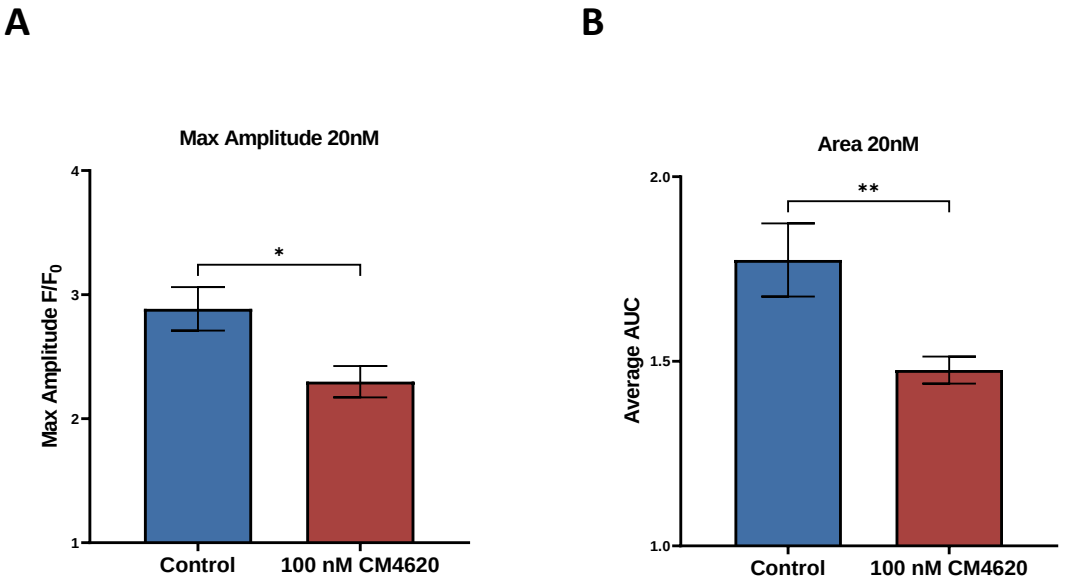



***Figure 5.5 Quantification of PACs during the application of low physiological doses of ACh to investigate the protective effects of CM4620.***

*Traces of shown in Fig. 5.4 were quantified to express the maximum amplitude of calcium signalling generated in addition to the average AUC during physiological ACh stimulation.* ***(A)*** *Maximum amplitude during the application of 20 nM ACh was calculated in cells either treated with a control Na+ HEPES buffer (n=7) or a treatment containing 100 nM of the CRAC channel inhibitor CM4620 (n=25). A significant reduction with a p-value of 0.0301 was observed due to the treatment with CM4620.* ***(B)*** *AUC during the application of 20 nM ACh was also calculated in cells which were either treated with a control Na+ HEPES buffer (n=7) or a treatment containing the CRAC channel inhibitor CM4620 (n=25). A significant reduction was also seen with a p-value of 0.0016 due to the treatment with 100 nM CM4620.*

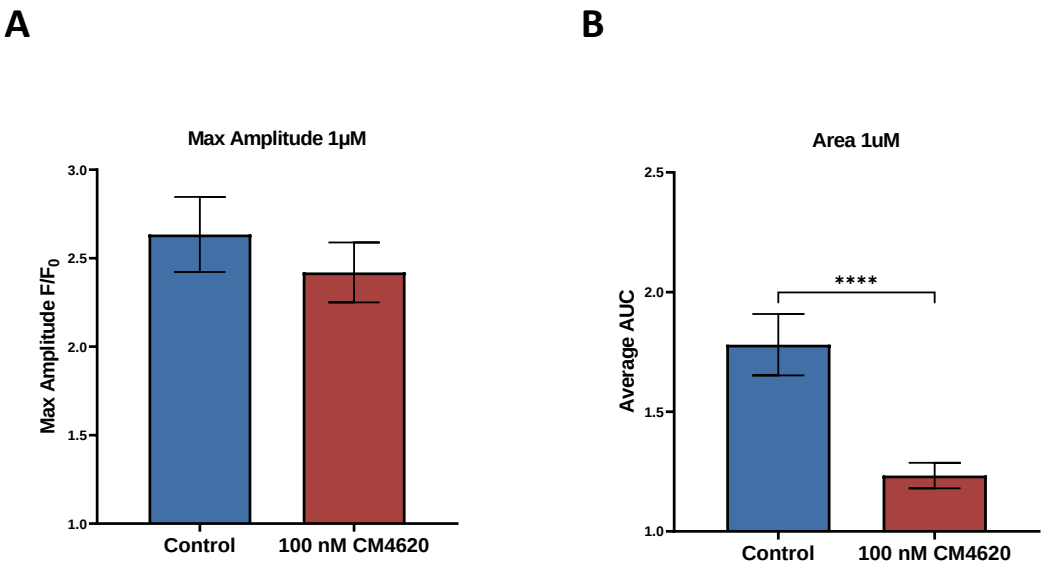



***Figure 5.6 Quantification of PACs during the application of high doses of ACh to investigate the protective effects of CM4620.***

*Traces of shown in Fig. 5.4 were quantified to express the maximum amplitude of calcium signalling generated in addition to the average AUC during a high concentration of ACh stimulation.* ***(A)*** *Maximum amplitude during the application of 1 µM ACh was calculated in cells either treated with a control Na+ HEPES buffer (n=7) or a treatment containing 100 nM of the CRAC channel inhibitor CM4620 (n=25). There was no observable statistical difference between the two treatments.* ***(B)*** *AUC during the application of 1 µM ACh was also calculated in cells which were either treated with a control Na+ HEPES buffer (n=7) or a treatment containing the CRAC channel inhibitor CM4620 (n=25). A significant reduction was observed with a p-value of <0.0001 due to the treatment with 100 nM CM4620.*

In addition to CM4620, other CRAC channel inhibitory compounds have been well studied and have provided vital information relating to SOCE in PACs. An example of this is GSK-7975A which is also a CRAC channel inhibitor that blocks Orai1 channels potentially downstream to STIM1/Orai1 interaction. However, further compounds have shown promising inhibitory results during calcium signalling. NED-19 is a cell permeable, non-competitive antagonist of NAADP. It has been well known that NED-19 can be used to disrupt calcium signalling involving NAADP. However, it is yet unknown as to whether NED-19 affects calcium entry in similar ways to that of GSK-7975A. Therefore, experiments to assess the impact of NED-19 upon calcium entry were proposed.

A representative control trace shown in Fig. 5.7 demonstrates the protocol that is carried out which contains the depletion of intracellular stores with CPA in a calcium free solution. After which, calcium entry is stimulated by applying external calcium. As demonstrated in Fig. 5.7, calcium enters the cytosol upon the application of $CaCl_2$ which provides extracellular free calcium ions. Following the removal of extracellular $CaCl_2$ (and consequently, extracellular calcium ions) cytosolic calcium extrusion is stimulated. Visibly comparing Fig. 5.8 to Fig 5.9 which applied 100 µM NED-19 simultaneously with extracellular calcium shows a markedly reduced entry of calcium. Furthermore, the protocol was repeated by substituting NED-19 with 100 µM GSK-7975A in order to compare the two compounds. Visually from the representative trace in Fig. 5.9, GSK-7975A is much more effective in disrupting calcium entry into the cell.

Once the maximum amplitudes of calcium entry were calculated and displayed in Fig 5.12 it is evident that GSK-7975A reduced calcium entry significantly more than NED-19. However, on the other hand, NED-19 also significantly reduced calcium entry when compared to control but not to the extent of GSK-7975A. The control mean of 2.002 ±0.0523 was reduced to 1.397 ±0.0394 with the application of NED-19 yielding a p- value of <0.0001. However, GSK-7975A also yielded a p-value of <0.0001 but reduced the maximum amplitude slightly more to 1.354 ±0.0328.

However, there was no statistical difference between GSK-7975A and NED-19 with a p-value of 0.9750.

Although there is minimal calcium entry during treatment with GSK-7975A, the entire calcium entry signal is not inhibited. Therefore, to investigate whether NED-19 and GSK-7975A target the same ORAI channels during calcium entry and whether the whole calcium entry signal could be blocked, it was proposed to combine the two compounds during incubation with the cells. The resulting calcium entry in Fig. 5.11 following the application of both NED-19 and GSK-7975A is significantly reduced, however, it is not completely abolished. This combinatory treatment produced a significant reduction with a mean of 1.291 ±0.0470 and an observed p-value of <0.0001 compared to the control dataset. Additionally, the combination of NED-19 and GSK-7975A did not yield any significant difference when compared to the sole application of NED-19 or GSK-7975A with p-values of 0.8091 and 0.9553 respectively.

Although the mechanism of action of GSK-7975A is relatively well known, the uncertainty regarding NAADP signalling, as previously discussed, poses questions about how NED-19 specifically inhibits calcium signalling. The resulting calcium entry during the combination of the two treatments potentially suggests that the two compounds may affect a common pathway. However, as there is a significant difference between the effects of the two compounds, it seems that NAADP signalling plays a much smaller role during SOCE.

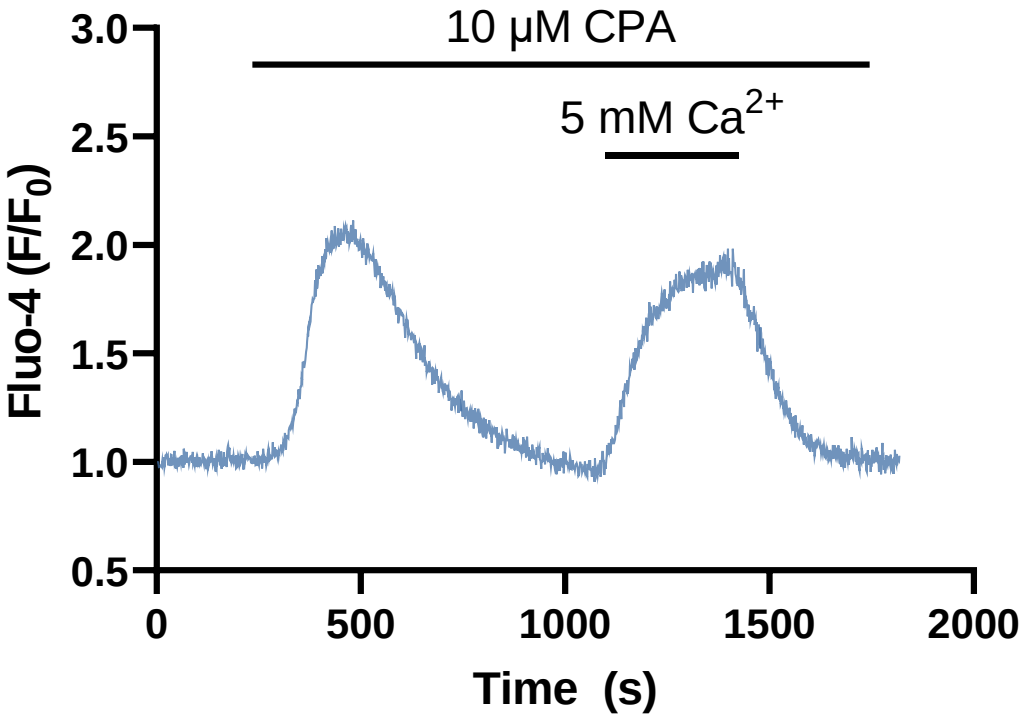



***Figure 5.7 Representative calcium entry trace of a control PAC Following CPA application.***

*Cells were treated with 10 µM CPA in a calcium-free solution. Following the depletion of intracellular calcium stores, cells were exposed to a 5 mM calcium solution which was then removed once the calcium entry had peaked (n=53).*

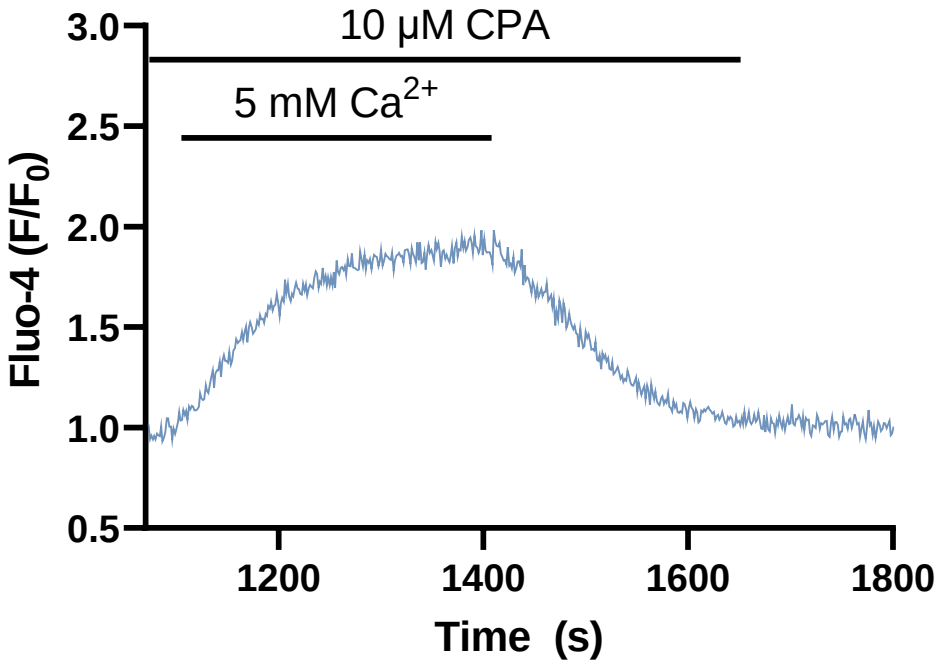



***Figure 5.8 Representative analytical calcium entry trace of a control PAC.*** *Shortened analytical region of calcium entry in cells which were treated with 10 µM CPA in a calcium-free solution. Following the depletion of intracellular calcium stores, cells were exposed to a 5 mM calcium solution which was then removed once the calcium entry had peaked. This trace is a representative of the control dataset (n=53).*

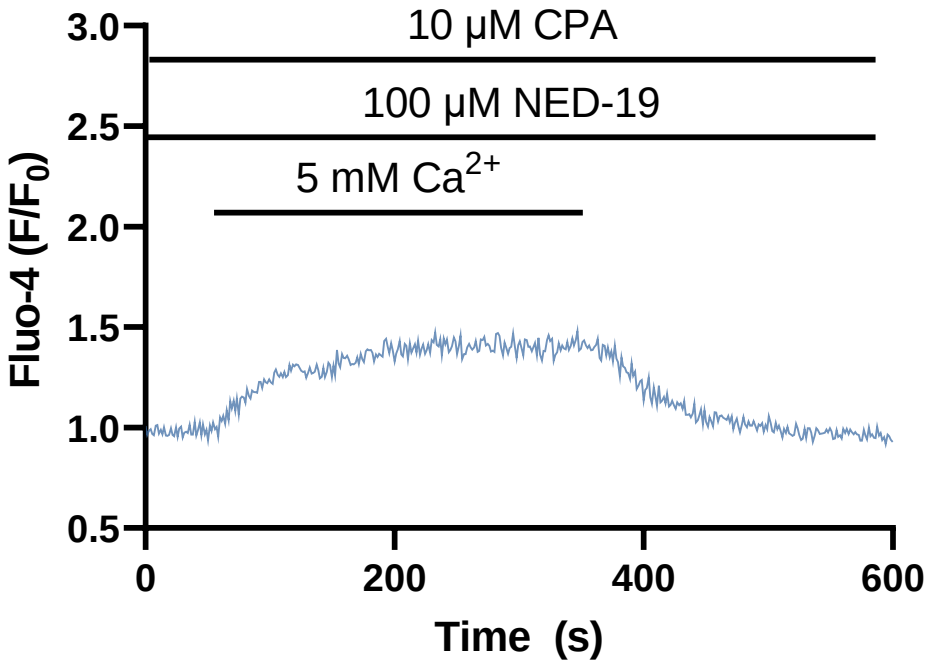



***Figure 5.9 Representative calcium entry trace of a NED-19 treated PAC following CPA application.***

*Cells were treated with 10 µM CPA in a calcium-free solution. Following the depletion of intracellular calcium stores, cells were incubated with 100 µM NED-19 10 minutes prior to being exposed to a 5 mM calcium solution also containing NED-19. The calcium containing solution was then removed once the calcium entry had peaked. This trace is a representative of the cells treated with 100 µM NED-19 (n=18).*

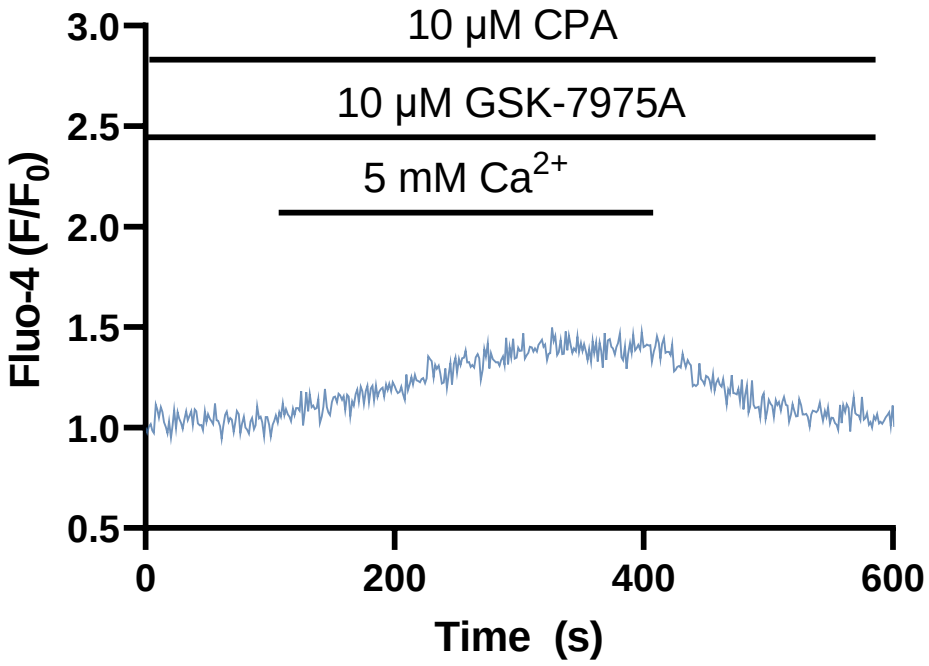



***Figure 5.10 Representative calcium entry trace of a GSK-7975A treated PAC following CPA application.***

*Cells were treated with 10 µM CPA in a calcium-free solution. Following the depletion of intracellular calcium stores, cells were incubated with 100 µM GSK-7975A 10 minutes prior to being exposed to a 5 mM calcium solution also containing GSK-7975A. The calcium containing solution was then removed once the calcium entry had peaked. This trace is a representative of the cells treated with 10 µM GSK-7975A (n=16).*

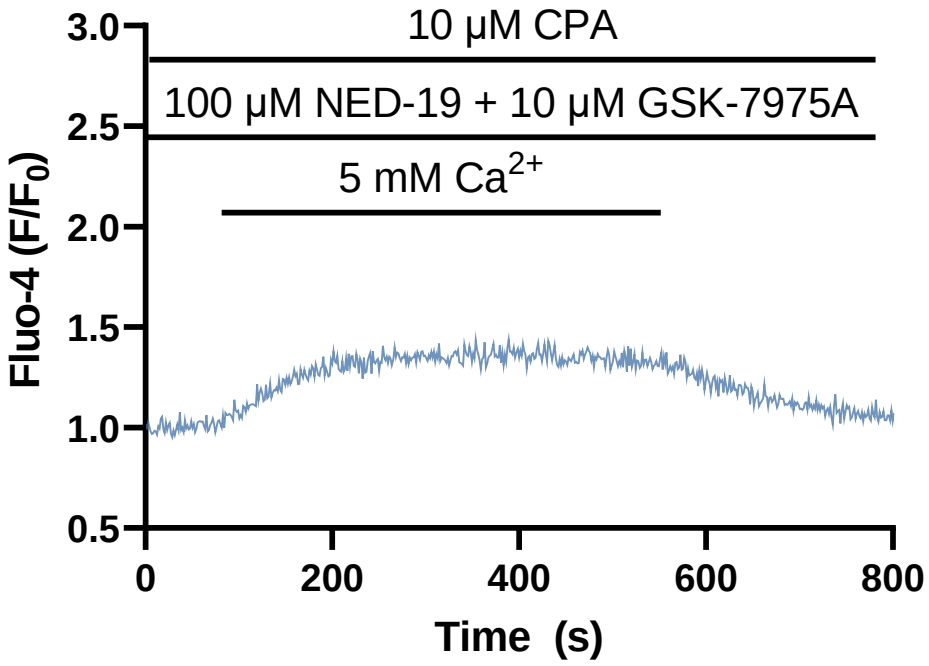



***Figure 5.11 Representative calcium entry trace of a PAC following CPA application and treatment with both NED-19 and GSK-7975A.***

*Cells were treated with 10 µM CPA in a calcium-free solution. Following the depletion of intracellular calcium stores, cells were incubated with 100 µM NED-19 and 100 µM GSK-7975A 10 minutes prior to being exposed to a 5 mM calcium solution also containing NED-19 and GSK-7975A. The calcium containing solution was then removed once the calcium entry had peaked. This trace is a representative of the cells treated with a combination of 100 µM NED-19 and 10 µM GSK-7975A (n=10).*

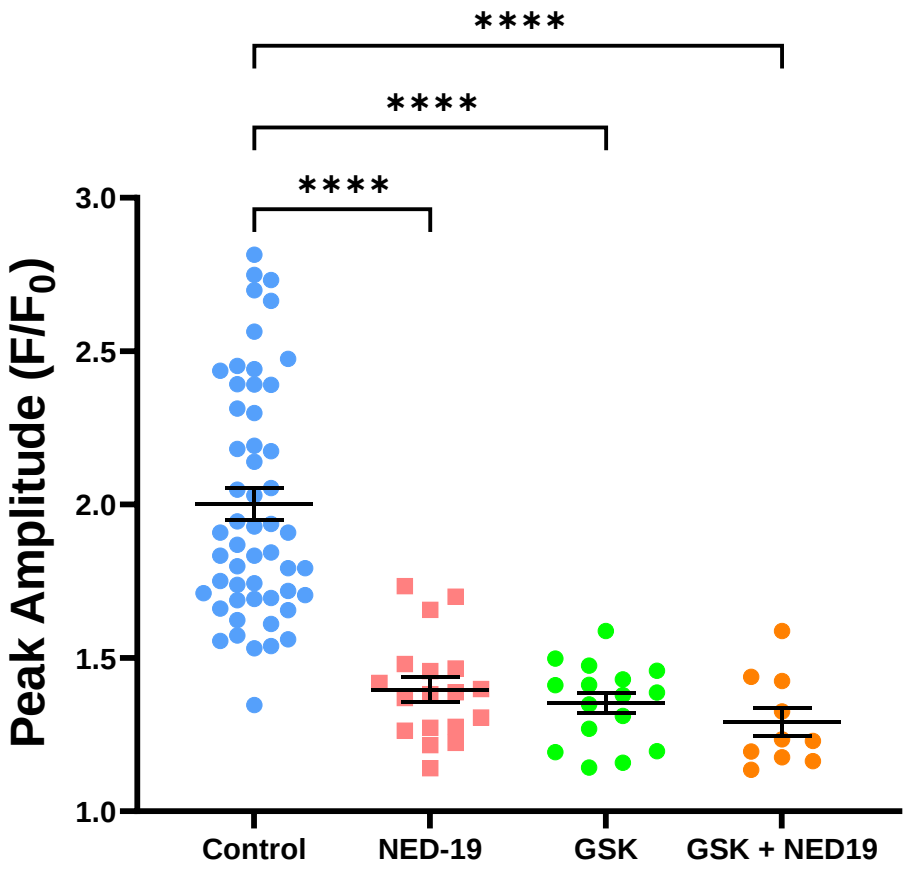



***Figure 5.12 Mean peak intracellular calcium amplitude during calcium entry following treatment of inhibitory compounds.***

*This figure collates the data represented in Figs. 5.7 - 5.11. The results of a One-Way ANOVA and a Post-Hoc Tukey's Multiple Comparison Test demonstrated that each treatment was significantly different to the control with the addition of a significant difference shown between NED-19 and GSK-7975A (* = P < 0.05, ** = P < 0.01, *** = P < 0.001).*

## 5.3 Discussion - Potential Therapeutics of Acute Pancreatitis Targeting Calcium Specific Channels

For years, scientists and companies have been in search for therapeutic compounds which could treat or prevent the development of acute pancreatitis. During this search, the knowledge of intracellular calcium signalling has provided mechanisms within the cell to target (34,35,170,297,338). Use of these inhibitory compounds has greatly benefitted research by allowing the isolation or prevention of specific mechanisms or targets to test certain hypotheses. The results that were found whilst investigating CM4620 upon ACh stimulation in Fig. 5.3-6 are good examples of this. The introduction of the CRAC channel inhibitor CM4620 demonstrated significant reductions in calcium signalling both during physiological oscillatory signalling patterns in addition to global pathophysiological signalling.

Although previously carried out studies of CM4620 inhibitory and protective effects during pathological signalling are extremely promising, the results found during physiological signalling induced by 20 nM ACh are just as informative as the pathophysiological signalling reduction. The reduced cytosolic signalling confirms that CM4620 is an extremely strong candidate for treating acute pancreatitis by not only reducing the overall area of the induced calcium signal, but also the maximal levels reached during signal induction, especially during lower concentrations of ACh stimulation. Furthermore, the results shown may also confirm the importance of CRAC channels during global sustained calcium signalling induced by 1 µM ACh. Although, there was no significant difference comparing maximum amplitudes between control and CM4620 traces, there was a significant difference observed when the AUC analysis was carried out in Fig. 5.6. This difference in observed statistical differences may suggest that CRACs play a greater role during the sustaining phase of calcium signals compared to their initiation which has been the suggested hypotheses for quite some time (35,159,163,164,197,228,345). Hence, the maximal amplitude during high ACh concentrations was not affected whilst the sustained calcium signal during this concentration was significantly reduced. Nevertheless, CM4620 is a promising drug which is currently undergoing clinical

trials with the data presented here strongly backing the recommendation to investigate the use of this compound more (341).

Following on from the CM4620 results, it's clear that the testing of inhibitory compounds can help determine the responsibility of certain receptors, mechanisms etc. during calcium signalling (34,35,170,297,338). Previous studies have utilised the NAADP antagonist, NED-19, to great effect in order to assess the involvement of NAADP (175,346). NED-19 has been used multiple times to inhibit calcium signals, especially those initiated by CCK or pathological agents. As it is still currently unknown how NAADP specifically binds to or activated RyRs during the initiation of calcium signalling it was questioned whether NAADP plays any role during SOCE. Therefore, NED-19 could provide some insight into whether this happens. As a consequence, a calcium entry protocol was devised to test the effect of NED-19 alongside other well-known CRAC inhibitors to assess NED-19's performance.

NED-19 was found to reduce calcium entry following the depletion of intracellular calcium stores. However, when comparisons were made between the effectiveness of NED-19 and GSK-7975A, a clear significant difference was seen. The sole use of GSK-7975A markedly reduced the influx of calcium more than that of NED-19. Furthermore, the combination of NED-19 and GSK-7975A treatment did not provide any further benefit to reducing the influx of calcium. This may be due to the high concentrations of the compounds used which could have produced the maximal level of inhibition shown. However, it also may not be unreasonable to assume that the two compounds may share the same inhibitory mechanism of action.

In addition to this, previous findings may also shed light upon the results found within this study. The secondary $Ca^{2+}$ messenger molecule, NAADP, has been closely associated with TPC mediated $Ca^{2+}$ release from acidic stores (177,198–200,202,347). The activation of TPC-mediated $Ca^{2+}$ release then induces SOCE via CICR. However, Lopez *et al.* (347) demonstrated the surprising association between TPC2 with STIM1 during $Ca^{2+}$ depletion, strongly suggesting that TPC2 plays a role in the regulation of SOCE. Furthermore, Lopez *et al.* (347) suggest that TPC2 may also

associate with Orai1, therefore, potentially implicating NAADP-mediated $Ca^{2+}$ signalling even more in its potential involvement in SOCE. Therefore, it cannot be out of the question that NED-19 inhibition of NAADP-mediated $Ca^{2+}$ release also inhibits SOCE due to TPC -Orai1 and STIM1 interaction. Supplementary evidence which may also support the evidence found here, is the close association between cADPR and NAADP-mediated $Ca^{2+}$ signalling (348). The multi-functional enzyme, CD38 plays a vital role in the conversion of NAD into cADPR or NAADP (349). Both of these have been implicated during calcium overload in pancreatic cells upon TLC-S application (176,350). In the past, this has primarily been thought to occur due to the activation of RyRs by NAADP and cADPR. However, there is move evidence suggesting the activation of TRPM2 by cADPR which leads to the direct movement of $Ca^{2+}$ ions into the cytosol from extracellular stores (348). Whether or not this also applies to NAADP remains yet to be confirmed. Moreover, the critical enzyme CD38 which is involved in the process of cADPR and NAADP formation has also provided evidence that NAADP-mediated calcium signalling may directly induce SOCE (349). The knockout of the CD38 enzyme from LAK cells indicated a reduction in SOCE which encourages the findings found within this study that an antagonist of NAADP also results in the inhibition of SOCE (349).

As a consequence of this study's findings, the experiments should be repeated with lowering and differing concentrations of NED-19 and GSK-7975A to confirm the effect of NED-19 on calcium entry. Conversely, it is unlikely given the inhibitory basis of NED-19 that it would directly affect ORAI channels. Taking this into account, the concluding hypothesis from these experiments may suggest that NAADP plays some sort of role during calcium entry which partially reduced calcium entry, particularly as there was no change in calcium entry reduction when combining the two treatments, NED-19 and GSK-7975A compared to only applying GSK-7975A.

# Chapter 6

# Discussion and Conclusions

## 6.1 Introduction

During the physiological life cycle of pancreatic cells, calcium signalling is extremely important and is required to carry out complex cellular functions. Ranging from the regulation of protein synthesis to zymogen and enzyme secretion. Not only are the cellular functions regulated by calcium, but calcium itself is regulated and controlled by a multitude of pathways which have been discovered whilst the pancreas has been studied for centuries. Although our knowledge of the physiological roles of the pancreas and its regulation is comprehensive, there are still many unknowns. Despite our understanding of $IP_3$ and NAADP mediated calcium signals, it is still unclear the exact mechanisms involved in RyR activation by NAADP.

Furthermore, it is evident that calcium signalling also plays a major role in pancreatic pathophysiology. Numerous studies have shown that calcium overload within pancreatic cells causes the development of acute pancreatitis (38). Differing cell types also play different roles during pathology and are uniquely affected by intracellular calcium overload (272,351). Within PACs, the premature activation of pro-enzymes and necrosis are triggered whilst PSCs become activated and express extracellular matrix proteins (279,297). Therefore, it is not unreasonable to assume that if these calcium overloading signals are prevented, the development of acute pancreatitis can be reduced.

During the majority of studies in previous years, a lot of emphasis has been placed upon the exact mechanisms involved in calcium signalling during pathology. This is so specific targets within those mechanisms can be identified for therapeutic intervention. Throughout this project, compounds and peptides were investigated in a multitude of experiments to measure their ability and potential use as therapeutics against the development of acute pancreatitis. Furthermore, not only does the study of these therapeutic agents shine light on the potential to prevent pathology, but also aids our ability to understand and investigate intracellular $Ca^{2+}$ signalling mechanisms.

## 6.2 Discussion - Conclusion

In overall conclusion, this project has produced some extremely interesting and promising results. Firstly, the novel investigation of BH4-Bcl-2 protection of mitochondrial calcium influx in response to physiological and pathological stimuli generates some insight into the mechanisms involved in mitochondrial calcium signalling and pathology. The further analysis of the pathophysiological signalling also highlighted a significant difference in the rate of the initial response. The BH4-Bcl-2 peptide provided some protection against pathological calcium signalling within mitochondria, most likely as a consequence of reducing cytosolic calcium levels during the signal's initiation.

Furthermore, not only was BH4-Bcl-2 confirmed as a strong regulator of cytosolic calcium signalling in PACs within this study, we produced evidence that the BH4-Bcl-2 peptide also provided protective effects within PSCs. Although this protection was not as great as that seen within PACs, this is the first time that the effects of BH4-Bcl-2 has been investigated within PSCs. Additionally, whilst investigating PSCs *in situ*, the ability to simultaneously investigate PACs *in situ* was presented. This also yielded similar results to studies that measured isolated PACs such as Vervliet *et al.* (329). However, the novel investigation into the effects of BH4-Bcl-2 *in situ* are extremely useful and demonstrate the potential use of BH4-Bcl-2 as a regulator of pathological calcium signalling. Finally, the introduction of the sugar galactose into the previous experiments also yielded extremely interesting results. In each experiment galactose provided additional protection against calcium signalling, especially within mitochondrial signalling and cellular necrosis. The addition of galactose alongside the use of BH4-Bcl-2 significantly reduced the rate of the initial response to TLC-S. Furthermore, galactose seemed to play a crucial role when in combination with BH4-Bcl-2 in protecting against necrosis. Although the pure combination of BH4-Bcl-2 and galactose did not yield significantly lower cell death than the sole application of BH4-Bcl-2. However, it enabled the reduction of the concentration of BH4-Bcl-2 which is particularly useful as this further reduces the volume of DMSO required in the final treatment which is essential.

This combinatory treatment of BH4-Bcl-2 and galactose was not only seen during TLC-S stimulation but also during asparaginase stimulation. Although it was no possible to continue the investigation of asparaginase-induced pathology and the protective effects of BH4-Bcl-2, this result suggests that BH4-Bcl-2 and galactose may be extremely beneficial in the battle against asparaginase-induced acute pancreatitis.

Throughout this study, strides have been made in assessing the effectiveness of the NAADP antagonist, NED-19, and the CRAC channel inhibitor, GSK-7975A, in preventing or reducing cytosolic calcium overload. Again, the novel application of NED-19 to a calcium entry protocol produced some intriguing findings which also triggers additional questions to the mechanisms that are involved in this inhibition. Although the mechanisms of NED-19 inhibition of NAADP-mediated calcium signalling in PACs is well documented, there is also little evidence suggesting mechanisms of inhibition with PSCs. Linking the newly found NED-19 inhibition of calcium entry, investigation into potential NED-19 driving inhibition in PSCs could provide key understanding in physiological and pathophysiological signalling within PSCs. The signalling mechanisms within PSCs are markedly different to neighbouring PACs mainly due to their size. As a consequence, calcium signalling within PSCs is much more reliant upon calcium entry as opposed to calcium release (36,269,272,279). Therefore, more experimentation is required to assess the direct mechanisms which are involved during NED-19 inhibition within PSCs.

NED-19 was found to reduce calcium entry following the depletion of intracellular calcium stores. However, when comparisons were made between the effectiveness of NED-19 and GSK-7975A no significant difference was produced. The sole use of GSK-7975A reduced the influx of calcium markedly more than that of NED-19 however, the combination of NED-19 and GSK-7975A treatment did not provide any significant benefit to reducing the influx of calcium. Therefore, it may be reasonable to assume that the two compounds may share the same inhibitory mechanism of action. On the other hand, it is unlikely given the inhibitory basis of NED-19 that it would directly affect ORAI channels. Taking this into account, the

concluding hypothesis from these experiments may suggest that NAADP plays some sort of role during calcium entry which partially reduced calcium entry, particularly as there was no change in calcium entry reduction when combining the two treatments, NED-19 and GSK-7975A compared to only applying each compound independently.

### 6.3 Future Directions

This thesis has utilised multiple novel techniques which has improved our knowledge on the potential uses and effects of the BH4-Bcl-2 peptide. The BH4-Bcl-2 peptide reduced and regulated mitochondrial calcium signalling during pathological and physiological signalling. Furthermore, the peptide greatly reduced cell death rates during pathological cell death assays. However, although these effects are extremely promising and build upon previously collected published findings regarding the protective effects of BH4-Bcl-2, this thesis does not provide specificity into the exact mechanisms that are affected during BH4-Bcl-2 calcium signalling regulation. Therefore, in future studies, the exact mechanisms in which BH4-Bcl-2 recruits to provide its protective effects should be explored. The reductions in mitochondrial calcium due to the presence of BH4-Bcl-2 may be due to reduced cytosolic calcium signalling. Previously carried out studies by *Vervliet et al.* (329) clearly demonstrated reductions in cytosolic calcium levels due to BH4-Bcl-2. Although the reductions in mitochondrial calcium seen in this study are evident, it cannot be assumed that these two effects caused by BH4-Bcl-2 are not linked. During mitochondrial calcium signalling, there is an influx of calcium from the cytosol into the mitochondria. Therefore, is logical to presume that if cytosolic calcium is reduced, then the influx of calcium into the mitochondria must also be reduced.

Building upon the results found in this thesis, there is strong evidence to suggest the potential use of the combination treatment of BH4-Bcl-2 and galactose. The novel reductions shown by this treatment during mitochondrial signalling are

extremely promising, especially given the further protection seen during necrosis. The ability to use a lower concentration of BH4-Bcl-2 by combining it with galactose is extremely exciting. By reducing the volume of DMSO required to dissolve BH4-Bcl-2, the more likely it is to be able to drive the use of BH4-Bcl-2 as a therapeutic alternative in the future. Therefore, future studies investigating the lowering of BH4-Bcl-2 concentrations in combination with galactose would be extremely beneficial. Additionally, the use of BH4-Bcl-2 and galactose in this thesis has clearly demonstrated protective effects against cell death and excessive mitochondrial calcium signalling, however, it would also be extremely beneficial to investigate the effects of these treatments have upon ATP levels within the cell. This would vastly increase our understanding of the effects of physiological stimuli upon pancreatic cells in addition to improving our knowledge of the mechanisms protected by BH4-Bcl-2.

Additionally, the results presented within this thesis strongly suggest investigating the use of BH4-Bcl-2 and its combination with galactose in future models targeting the development of pancreatic pathology. Therefore, it would be hoped that the *ex vivo* results found here could be replicated in an *in vivo* model. This would be a critical next step to proposing BH4 and galactose as viable therapeutic strategies in the treatment of acute pancreatitis.

### 6.4 Limitations

At the inception of this thesis, it was planned to investigate the effects of pathological stimuli including TLC-S and asparaginase upon cytosolic and mitochondrial calcium in PSCs. Furthermore, it was initially intended to investigate the possible protective properties BH4-Bcl-2 and galactose would have had upon these effects. However, due to unforeseen circumstances including COVID-19, these planned experiments were unable to be completed. Therefore, it would be of great interest to carry out these experiments in any future studies on the BH4-Bcl-2 peptide.

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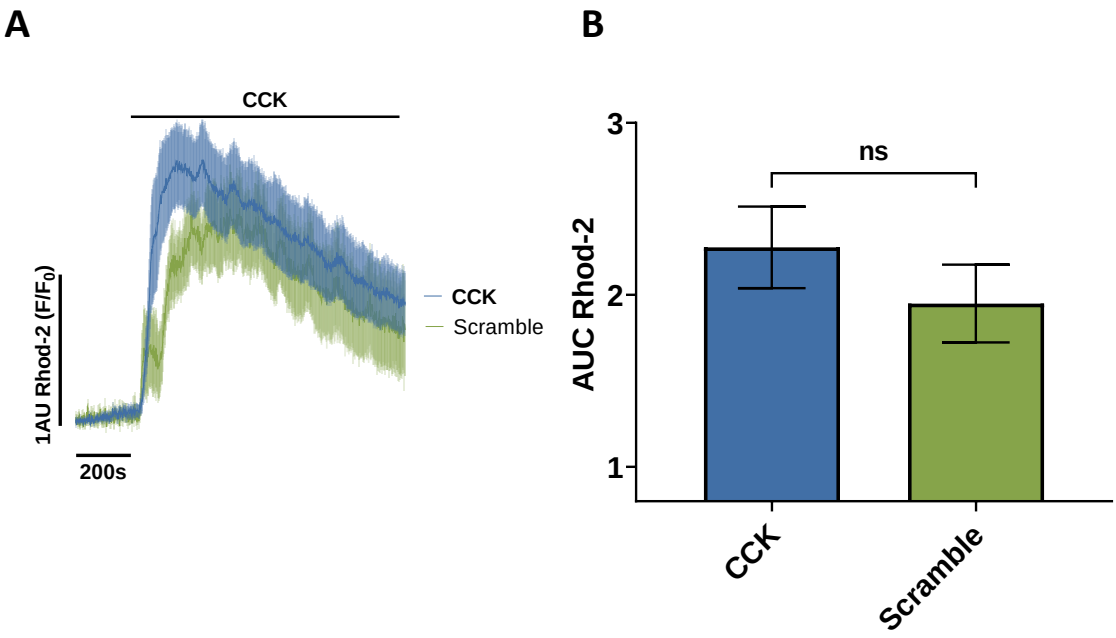
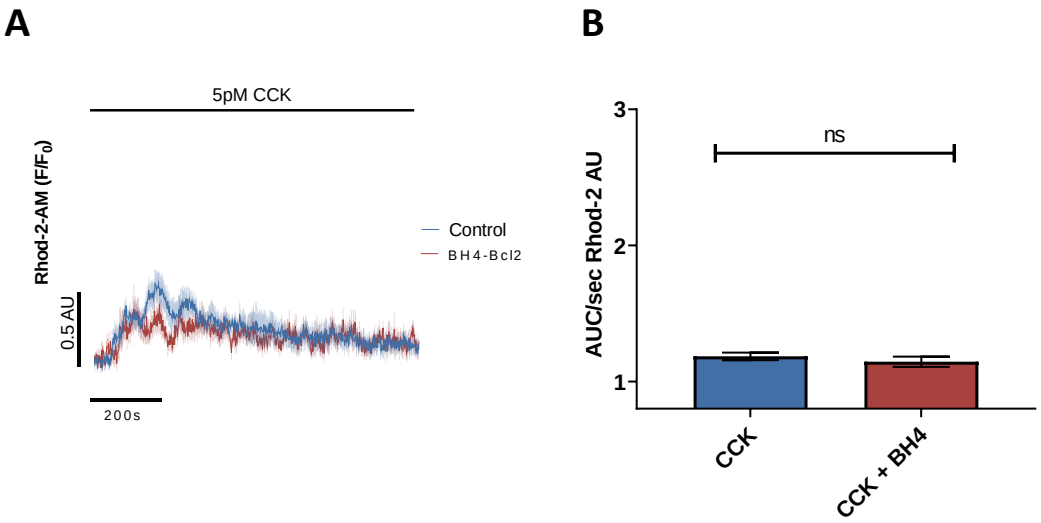
# Appendix



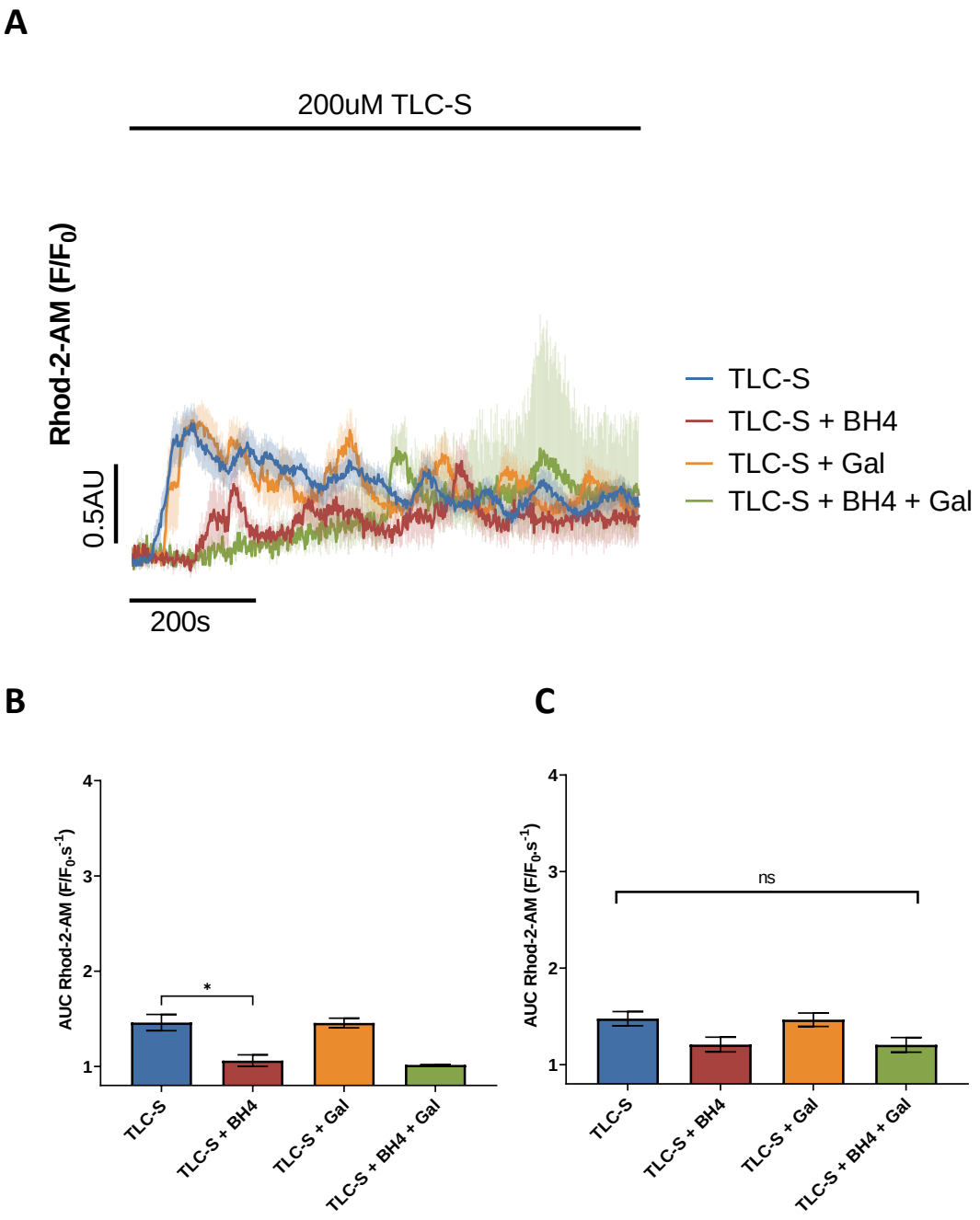

***Appendix 1 Mitochondrial calcium analysis of isolated PACs in response to CCK stimulation.***

*Average mitochondrial calcium traces of isolated Rhod-2-AM loaded PACs in response to 5pM CCK stimulation.* ***(A)*** *Cells were either untreated controls with DMSO addition (n=12) or treated with 50 µM BH4-Bcl-2 peptide (n=10). Average traces were plotted with corresponding ±SEM values for mitochondrial traces. Cells were pre-incubated for 15mins with respective treatments and 5pM CCK was applied at 200 seconds. Buffer in every experiment contained 2mg/10mls of trypsin and chymotrypsin inhibitor.* ***(B)*** *The mitochondrial traces shown in (A) were quantified with total AUC.$s^{-1}$ values calculated from 200s to 1050s from start of experiment and plotted with ±SEM. A significant difference was observed between the control application of CCK with an average of 2.277 ±0.2372 and the application of 50 µM BH4-Bcl-2 peptide with an average value of 1.522 ±0.0721. An observed P-Value of 0.0110 was calculated between the two groups.*




***Appendix 2 Quantitative analysis of cytosolic calcium of isolated PACs in response to CCK stimulation.***

***(A)*** *Average cytosolic calcium traces of isolated PACs in response to 5pM CCK stimulation. Cells were either untreated control with DMSO addition (n=12), or treated with 50 µM BH4-Bcl-2 peptide (n=10). Average traces were plotted with corresponding ±SEM values for cytosolic traces shown in (A). Cells were pre-incubated for 15 mins with respective treatments and 5 pM CCK was applied at 200 seconds. Buffer in every experiment contained 2 mg/10mls of trypsin and chymotrypsin inhibitor.* ***(B)*** *The average AUC.s$^{-1}$ values for the total area of responses in (A) were quantified. Total AUC.s$^{-1}$ values calculated from 200s to 1050s and plotted with ±SEM. There were no significant differences shown between the control CCK dataset with a mean of 1.185 ±0.0277 and the BH4 dataset with a mean of 1.145 ±0.0380. An observed p-value of 0.1727 was produced. (* = P-Value <0.05, ** = P-Value <0.01, *** = P-Value <0.001, **** = P-Value <0.0001).*




***Appendix 3 Quantitative analysis of cytosolic calcium of isolated PACs in response to TLC-S stimulation.***

*Average cytosolic traces of isolated Rhod-2-AM loaded PACs in response to 200 µM TLC-S stimulation. Measurements were taken simultaneously as those shown in Fig. 3.3. Cells were either untreated controls with DMSO addition (n=15), treated with 50 µM BH4-Bcl2 peptide (n=7), treated with 1 mM Galactose with no peptide present*

*(n=6) or a combination treatment of 50 µM of a BH4-Bcl2 peptide and 1 mM Galactose (n=5). Average traces were plotted with corresponding ±SEM values for cytosolic traces shown in **(A)**. Cells were pre-incubated for 15mins with respective treatments and 200 µM TLC-S was applied at 200 seconds. Buffer in every experiment contained 2mg/10mls of trypsin and chymotrypsin inhibitor. The average AUC.s-1 values for the peak plateau area of responses were quantified in **(B)** for cytosolic traces shown in (A). Average AUC.s-1 values for this region were calculated from 350s to 600s from the start of recording and plotted with ±SEM. Statistical differences were only observed when comparing cytosolic traces. There was only one statistical difference seen between the Control dataset and 50 µM BH4 with a P-Value of 0.040. The average AUC.s-1 values for the total area of responses were quantified in **(C)** for cytosolic traces shown in (a). Total AUC.s-1 values were calculated from 200s to 1050s from start of experiment and plotted with ±SEM. Again, here there were no statistical differences observed between any datasets (* = P-Value <0.05, ** = P-Value <0.01, *** = P-Value <0.001, **** = P-Value <0.0001).*