

**Physiological and Pathological Intracellular
Calcium Release in Human and Murine
Pancreatic Acinar Cells**

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**In loving memory of my mother,
Eileen Murphy**

Physiological and Pathological Intracellular Calcium Release

in Human and Murine Pancreatic Acinar Cells

PhD Thesis Abstract

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Sustained, toxic elevations of pancreatic acinar cell cytosolic free calcium ion concentration ($[Ca^{2+}]_c$), such as those observed with supramaximal secretagogue stimulation (CCK) are implicated in acute pancreatitis. However, Cholecystokinin (CCK) has been thought to act only indirectly on human pancreatic acinar cells via vagal nerve stimulation, rather than by direct CCK receptor activation as observed in rodent pancreatic acinar cells. However, in the series of experiments presented here using human pancreatic acinar cells, CCK at physiological concentrations (1-20 pM) elicited rapid, robust, oscillatory rises of the cytosolic Ca^{2+} ion concentration ($[Ca^{2+}]_c$), showing apical to basal progression in acinar cells, in the presence of atropine and tetrodotoxin. The $[Ca^{2+}]_c$ rises were followed by increases in mitochondrial ATP production and secretion, concluding that CCK acts directly on acinar cells in the human pancreas.

The earliest pathological mechanisms, such as sustained, toxic elevations of the acinar cytosolic free calcium ion concentration ($[Ca^{2+}]_c$), incriminated in experimental pancreatitis have been previously demonstrated by non-oxidative metabolites of ethanol (FAEE's), as well as bile salts, at supramaximal concentrations. However, in the clinical situation such hyperstimulation is unlikely to occur. To simulate a more clinically relevant stimulus, pancreatic acinar cells were stimulated with lower doses of FAEE's and/or bile salts in combination with physiological doses of secretagogues - a process which may precipitate pancreatitis clinically.

Illustrated here, the toxic transformation of secretagogue induced physiological Ca^{2+} signalling occurs with the perfusion of low doses of TLCS and POAEE resulting in cell injury. The intracellular second messengers implicated are IP_3 , cADPR and NAADP with the IP_3 receptor channel pivotal with both toxins. However, as previously demonstrated with supramaximal concentrations of POAEE, if supplementary ATP is added to the intracellular milieu, cellular injury is avoided with continued extrusion of large quantities of Ca^{2+} from the cytosol indicating functional Ca^{2+} ATPase pumps. This is not observed in cells which do not receive supplementary ATP. The toxic sustained Ca^{2+} elevation is also prevented by the removal of external Ca^{2+} or blockade of IP_3 receptor using caffeine and cell injury is again avoided. Therefore, it may be concluded, that it is the large, sustained toxic $[Ca^{2+}]_c$ load which impairs mitochondrial function and ATP production leading to Ca^{2+} ATPase pump failure and ultimately cell death. Lowering sustained intracellular $[Ca^{2+}]_c$ by blockade of IP_3 receptor channels may reduce cell injury in clinical acute pancreatitis.

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Abbreviations

ACh	acetylcholine
ADP	adenosine diphosphate
AM	acetylmethoxy ester
ATP	adenosine triphosphate
BAPTA	1,2-bis(<i>O</i> -aminophenoxy)ethane- <i>N,N,N',N'</i> -tetraacetic acid
BC	Before Christ
BZiPAR	rhodamine 110, bis – (CBZ – L- isoleucyl – L –propyl – L – arginine amide) dihydrochloride
Ca ²⁺	calcium
[Ca ²⁺] _{cyto}	cytosolic free Ca ²⁺ ion concentration
[Ca ²⁺] _i	cytosolic free Ca ²⁺ ion concentration
cADPr	cyclic ADP – ribose
CCCP	carbonyl cyanide <i>m</i> – chlorophenylhydrazone
CCK	cholecystokinin
CICR	Ca ²⁺ induced Ca ²⁺ release
CT	Computerised Tomography
DAG	1,2 – diacyl – glycerol
EGTA	ethylene glycol-bis (β -aminoethyl ether)- <i>N,N,N',N'</i> -tetraacetic acid
ER	endoplasmic reticulum
ERCP	Endoscopic Retrograde Cholangiopancreatography

ETC	Electron Transport Chain
FA	fatty acid
FAEE	fatty acid ethyl ester
GFP	green fluorescent protein
GRP	gastrin releasing peptide
IP ₃	inositol 1,4,5 – trisphosphate
IP ₃ R	inositol 1,4,5 – trisphosphate receptor
kDa	kilodaltons
Mg ²⁺	magnesium
[Mg ²⁺] _{cyt}	cytosolic free Mg ²⁺ ion concentration
mOsM	milliosmolar
MTP	mitochondrial permeability transition
MPTP	mitochondrial permeability transition pores
NAADP	nicotinic acid adenine dinucleotide phosphate
NAADPR	nicotinic acid adenine dinucleotide phosphate receptor
NADH	reduced nicotinic adenine dinucleotide
NO	nitric oxide
PIP ₂	phosphatidylinositol 4,5 – bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMCA	plasma membrane Ca ²⁺ ATPase
PMT	photomultiplier tube
O ₂	oxygen

OA	palmitoleic acid
POAEE	palmitoleic acid ethyl ester
PTP	permeability transition pore
ROS	reactive oxygen species
RyR	ryanodine receptor
SERCA	sarco/endoplasmic reticulum Ca ²⁺ ATPase
SOC	store operated Ca ²⁺ channel
SR	sarcoplasmic reticulum
TLCS	tauroolithocholate sulphate
TTX	tetrodotoxin
UV	ultraviolet
VIP	vasoactive intestinal peptide
$\Delta\psi_m$	mitochondrial membrane potential

Chapter 1

Introduction

Acute Pancreatitis

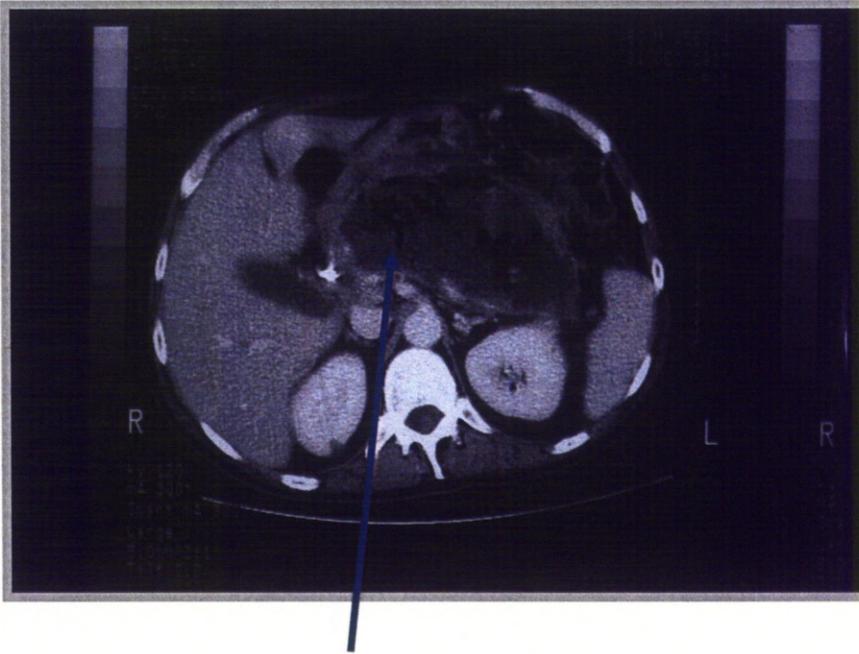
Acute Pancreatitis is an acute inflammatory, autodigestive condition of the pancreas and has an incidence of 150 – 450 /100,000 per year in the UK and is said to be increasing (Working Party on Acute Pancreatitis, 2005). It is a disease process, which often has severe complications requiring intensive care support in 20 – 30% of cases and has an overall mortality of 6 – 10% which has been static for last the two decades or so.

Previous improvement in mortality is attributed to several factors, such as better definition of the disease, faster identification of the need for critical care support, high quality imaging to identify complications and treatment at specialist centres (Neoptolemos et al, 1998)(figures 1.1, 1.2). But the majority of advances made have been in supportive therapy and treatment of complications arising from the condition.

There have been numerous randomised trials, which have largely failed to show a benefit of any particular treatment, and negligible advances in halting disease progression on first diagnosis, which would possibly hold the best prospect of decreasing morbidity and mortality in the future (Neoptolemos et al, 1998). To do this, it is imperative that we understand, in detail, the earliest cellular pathophysiological events so that more effective targeted treatments can be developed.

Several precipitating factors have been established for many years, such as gallstone disease discovered at the turn of the twentieth century (Opie et al, 1901), excess ethanol intake and hypercalcaemia (table 1.1). However, little progress has been made in altering the natural course of the disease process from first presentation aside from better supportive care.

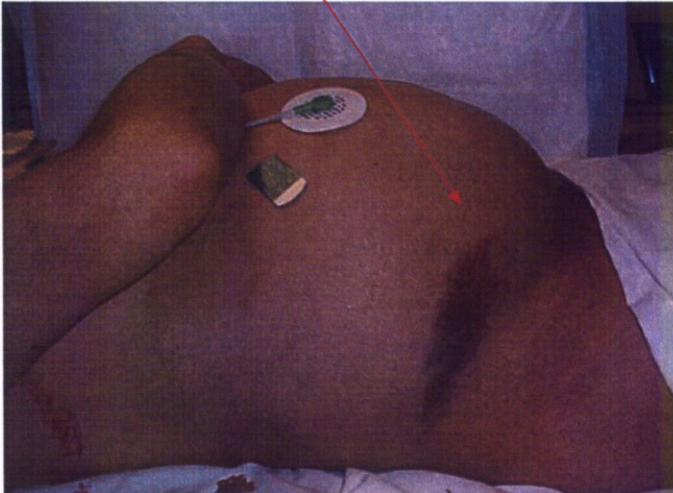
Figure 1.1



Cross sectional contrast-enhanced computerised tomography scan of upper abdomen of patient with pancreatic necrosis, showing the interior of the pancreas that is swollen and almost completely necrotic. Contrast has been injected intravenously and highlights living organs and tissues, including the liver and kidneys, but not the pancreas

Figure 1.2: Classical examples of a patient demonstrating haemorrhagic severe necrotising pancreatitis with retroperitoneal haemorrhage:

(a) **Grey Turners** sign of bruising in the flanks



(b) **Cullen's** sign of bruising around the umbilicus

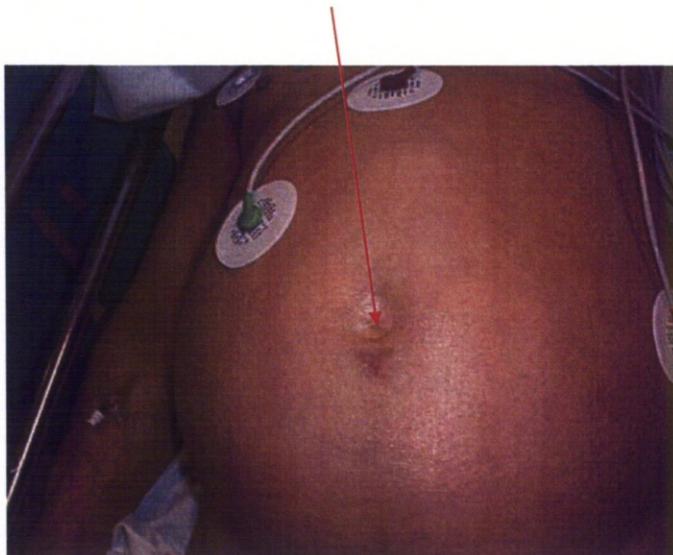


Table 1.1: Aetiologies of acute pancreatitis

Mechanical (Obstruction)	Cholelithiasis Gastric/biliary surgery Trauma Endoscopic Retrograde Cholangio Pancreatography Malignancy Ascaris infection Duodenal Obstruction
Metabolic	Alcohol Hypercalcaemia Hyperlipidaemia Drugs, e.g. thiazides, steroids, azathioprine Trinidadian scorpion venom (anticholinesterase)
Infective	Coxsackie B virus Mumps Cytomegalovirus Human Immunodeficiency Virus
Vascular	Cardio-pulmonary Bypass Mesenteric Ischemia/ embolism Peri-arteritis nodosa Hypothermia
Genetic	Hereditary Pancreatitis
Unknown	Idiopathic

Current treatment strategies in acute pancreatitis

Modern medical therapies in the treatment of disease progression once severe complicated acute pancreatitis ensues are by and large, wholly unacceptable.

Treatment during the acute episode can be broadly split into two major objectives, those being:

- 1) Supportive measures through the acute episode including intensive and invasive circulatory support, usually within the first two weeks of the disease process (figure 1.2)
- 2) Treatment of complications of the disease process itself, usually, but not necessarily occurring two weeks or more into its course.

Some advances in modern medicine and medical interventions have been astounding and fast paced in recent times in a wide variety of subject fields which has ultimately led to a greater life expectancy in the Western World, eg. Breast cancer screening leading associated increase in long term survival (Benson et al, 2009). In acute pancreatitis, however, the advances have been, at best, limited if considering targeted strategies at halting disease progression. Several randomised studies have failed to prove any benefit in a variety of treatments aimed at curtailing different pathways in an attempt to prevent disease progression (Fikri et al, 2003).

In the early stages in the disease process, one of the greatest challenges to surgeons and physicians, young or old is predicting which patient will run a short self limiting disease course with no subsequent long term morbidity, or, the patient who will

experience a severe necrotizing form of the disease resulting in significant morbidity or mortality (figure 1.1).

Ultimately, the reason why this is such a challenge, is to identify which patient will need intensive therapy support including intensive monitoring, invasive circulatory support and so forth in an attempt to avoid early death within the first two weeks.

The major issue is that no effective therapies to halt disease progression exists and there are subgroups of patients who, no matter how invasive and intensive the support, will succumb to the disease and mortality ensues regardless within the first few days or week. It is this group of patients in whom the study into the pathogenesis of the disease is thought will benefit the greatest with specific directed therapeutic targets.

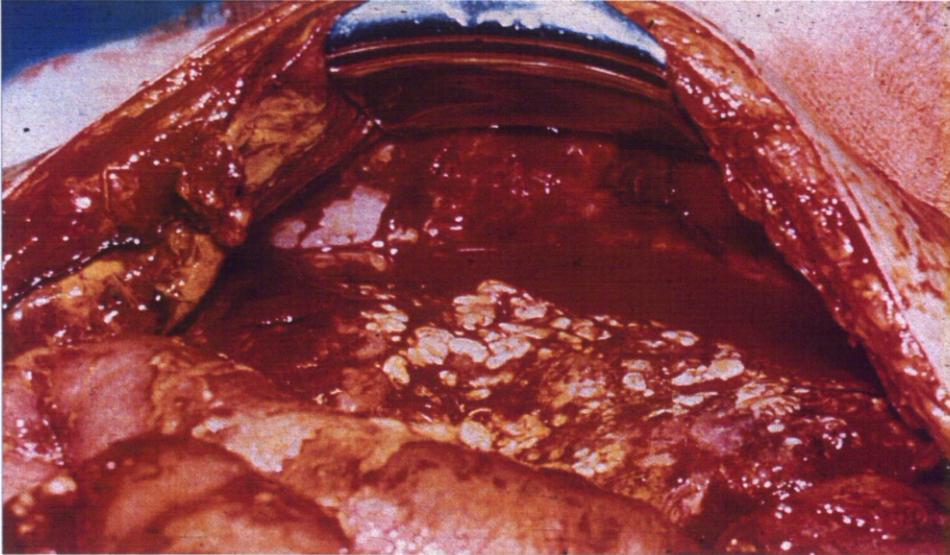
This concept may be of surprise in modern day medical circles as there is possibly a perception that death within the first week of severe acute pancreatitis is a rarity. But several audits carried out years ago have suggested that the mortality rate has changed little over the recent decades and 50-60 percent of deaths occurred during the first week of illness, usually as a consequence of multi organ dysfunction syndrome (MODS) (McKay et al, 1999). Of the early deaths, as many as eighty percent occurred within the first seventy two hours of admission with the patient most likely not transferred from the admitting hospital to a regional centre (McKay et al, 1999).

In patients who experience a severe episode of acute pancreatitis but survive the initial phase, it is the consequences and complications from the disease process that cause significant morbidity and mortality. This is the subgroup of patients who develop complications in and around the pancreas substance itself such as pancreatic necrosis, pancreatic pseudocyst formation, pancreatic abscess etc (figure 1.3).

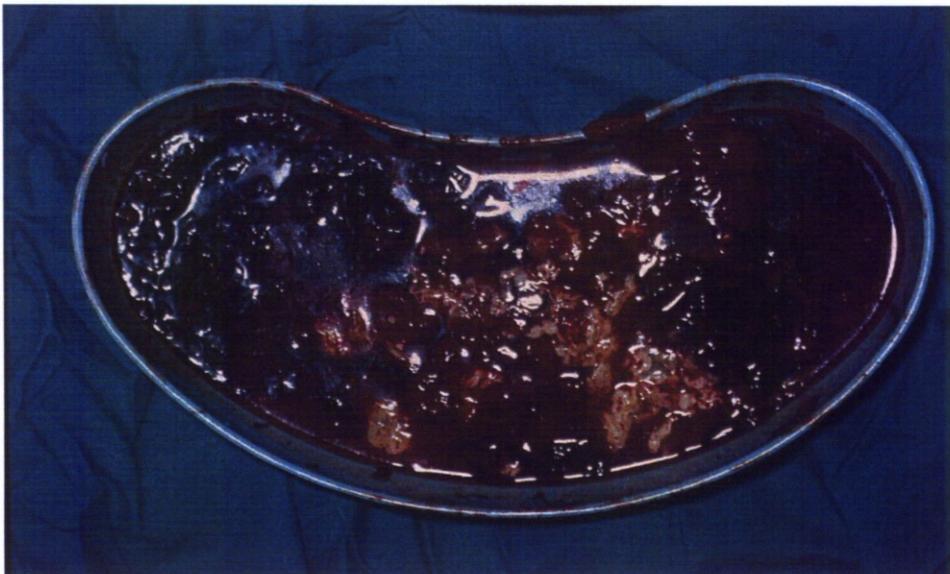
However, it is in this group of patients, there has been a great deal of progress in the last decade, which has resulted in significant reductions in mortality, and morbidity associated with the sequelae of the disease process.

Figure 1.3

Haemorrhagic necrotizing pancreatitis demonstrated at laparotomy



The portion of necrotic pancreas resected



Multi organ dysfunction syndrome (MODS) in acute pancreatitis

Early MODS in the first week is the major cause of death and causes major morbidity and a strain on resources (Buter et al, 2002). In the majority of cases the earliest target organ of the inflammatory response is the respiratory system. This commonly results in patients needing mechanical ventilatory support with increasing oxygen requirements as the adult respiratory distress syndrome (ARDS) is apparent resulting from lymphocyte infiltrate etc, in the alveolar tissue (figure 1.4).

From this point, if early organ dysfunction resolves it has no significant impact on mortality but significant morbidity and mortality results from the progression of the systemic inflammatory response (SIRS) to MODS with hepatic, renal and eventually circulatory failure (Buter et al, 2002).

Attempts to halt the SIRS at an early stage have been of great interest in the clinical research field, but have largely failed to produce any meaningful treatment in the clinical trial. A good example of this is the use of lexipafant, a platelet activating factor antagonist (PAF) as PAF is believed to amplify the activity of key mediators of SIRS. Despite lexipafant showing promise in preclinical and phase II clinical trials, the large randomised double blind multicentre study showed lexipafant had no benefits or effect on organ failure during treatment for severe acute pancreatitis (Johnson et al, 2001, McKay et al 2004, Imrie et al 1999). Other large trials such as those using octreotide, a somatostatin to reduce pancreatic secretions, also proved no benefit in clinical trials (Uhl et al, 1999, Buter et al, 2002).

This is probably as the disease process becomes complex and multifocal, whilst inhibiting one part of the inflammatory process does not have an overall effect and the

several other inflammatory mechanisms activated which are not being inhibited. From this we must use those antagonists targeting the initiation of the disease from its earliest point which may be effective in halting disease progression, or by targeting the inflammatory response at several points at the same time may be a more effective measure – a treatment which may be very difficult to administer.

Figure 1.4



Patient with multi organ dysfunction syndrome (MODS) secondary to severe acute pancreatitis. The patient is on an Intensive Therapy Unit, during the first week following onset of symptoms.

Invasive treatments in severe necrotizing acute pancreatitis

10-20% of patients develop a severe episode of acute pancreatitis, and those patients who survive the initial phase, marked by the systemic inflammatory response syndrome (SIRS) and multi organ dysfunction syndrome (MODS), are at very high risk of developing necrotizing pancreatitis (Buchler et al, 2000, Beger et al, 1986, Gerzof et al, 1987).

Significant pancreatic necrosis occurs in approximately 5-10% of patients which usually becomes apparent during the second and third week from initiation of the disease process (Winslet et al, 1992). The need for surgical intervention usually is determined by whether the pancreatic necrosis is infected or not. Sterile pancreatic necrosis is usually managed conservatively with resolution over a period of months and the patient improves with no continuation of the systemic inflammatory response and multi organ dysfunction syndrome (Connor et al, 2003, Rau et al, 1995, Karimgani et al, 1992).

The most common indication for surgery lies with evidence of infected pancreatic necrosis, usually identified by fine needle aspiration or extra intestinal gas on computerised tomography scanning (IAP guidelines for the surgical management of acute pancreatitis, 2003).

The aim of surgical intervention is eventual removal of all necrotic tissue, providing free drainage to facilitate resolution of the infected pancreatic collections (Lee et al, 1992).

The traditional surgical procedure of pancreatic necrosectomy is by the open technique (via a laparotomy) but newer techniques have been developed broadly fitting into three categories:

- 1) Open Necrosectomy
- 2) Percutaneous radiologically controlled procedures
- 3) Minimally Invasive Retroperitoneal Pancreatic Necrosectomy

Open Necrosectomy

Open necrosectomy is the traditional 'gold standard' procedure employed for decades now (figure 1.3). There are several variations including:

- 1) Open necrosectomy with closed lavage of the retroperitoneum (Buchler et al, 2000, Beger et al, 1998).
- 2) Open necrosectomy with multiple drainage and subsequent re-laparotomy as per requirement (Rattner et al, 1992, Fernandez-del Castillo et al, 1998)
- 3) Planned recurrent open necrosectomy and laparostomies and recurrent debridements (Fugger et al, 1991)
- 4) Open necrosectomy in stages with re-laparotomies and followed by delayed primary closure and drainage (Sarr et al, 1991, Tsiotos et al, 1998)
- 5) Open necrosectomy and open packing with re-laparotomies (Bradley et al, 1993, Orlando et al, 1993, Branum et al, 1998).

These approaches are usually associated with a mortality rate of between 25-50%. In an attempt to reduce the mortality rates experienced with open necrosectomy, minimally

invasive techniques' have been developed to reduce the overall traumatic injury incurred during recurrent laparotomies.

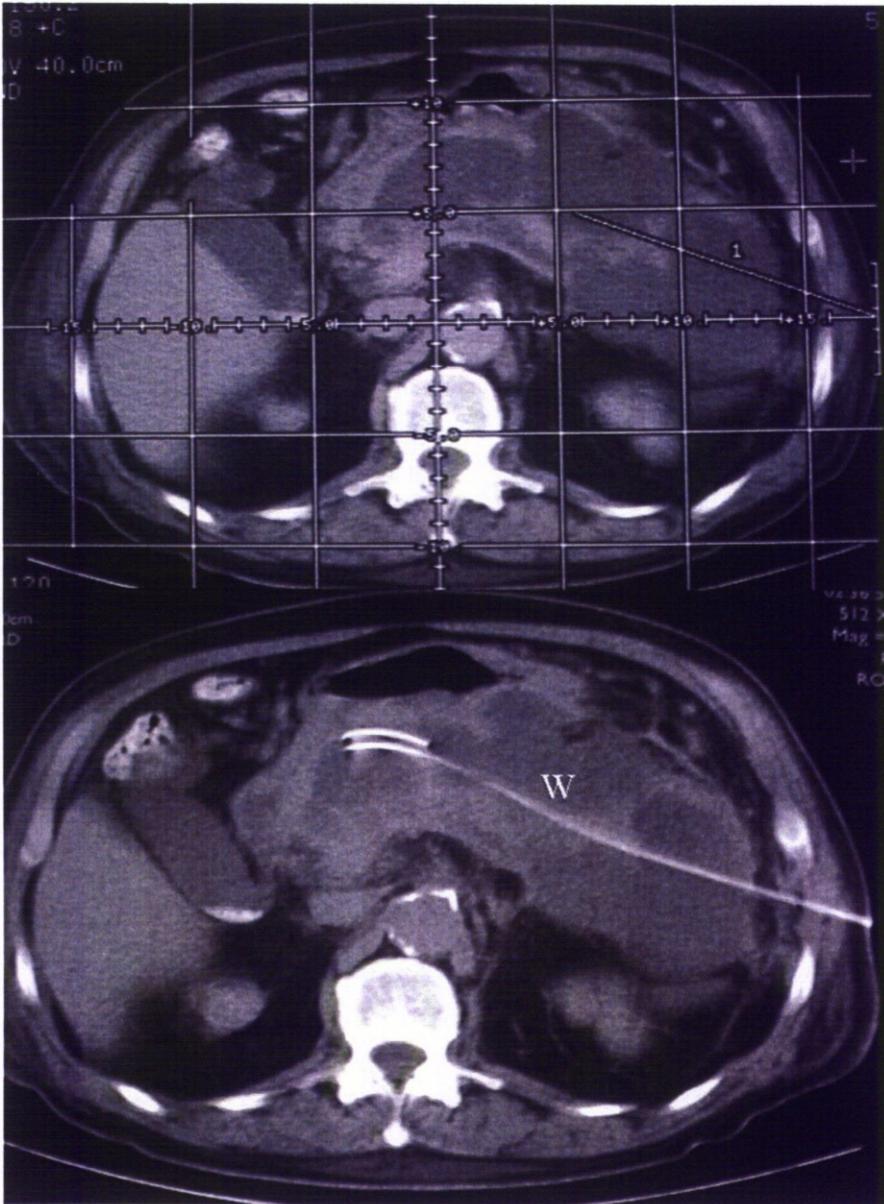
Percutaneous radiologically guided necrosectomy

Percutaneous radiological drainage of pancreatic collections requires the insertion of large bore drains on computerised tomography guidance (usually up to 32g French drains) and allowing the collections to drain over a period of time (figure 1.5).

Although percutaneous radiological drainage has been reported to be successful in some selected pancreatic collections, the technique is usually unsuccessful in patients with substantial pancreatic necrosis as the material substance itself is of a thick viscous nature that needs to be evacuated manually and so is only used in very selective circumstances if at all (Van Sonnenburg et al, 1985, Lee et al, 1992, Echenique et al, 1998, Freeny et al, 1998). Other such techniques using endoscopic means have also been introduced recently with access to the necrotic debris being made via an endoscopic gastrostomy and resection with lavage being performed through this. Further evidence of the efficacy of such techniques has yet to be reported.

Figure 1.5

Percutaneous Guidewire Insertion for radiological drainage of pancreatic necrosis



Minimally invasive pancreatic necrosectomy

Minimally invasive pancreatic necrosectomy was originally designed and used in Glasgow in the late 1990's and subsequently developed by the Liverpool group after tuition from the pioneers from Glasgow (Carter et al, 2001, Connor et al, 2003). The technique involves the insertion of a 12g French catheter under CT guidance via the left loin avoiding the pleural cavity, left kidney, spleen and the left and transverse colon. The tract is then dilated using renal dilators under direct x-ray screening.

The tract will then allow passage of a rigid cystoscope and debris can be removed under direct vision using forceps. When all loose material has been removed continuous irrigation is performed using modified intercostal chest tubes/drains. The procedure is repeated as many times as needed until all necrotic debris is removed (Connor et al, 2003). The procedure does not require general anaesthesia in the majority of occasions with local anaesthesia and sedation adequate in most situations.

Although the minimally invasive techniques have resulted in longer inpatient hospital stay, there has been a significant reduction in mortality from the procedure and other groups around the world are adopting similar techniques in order to attempt to reduce their mortality associated with infected pancreatic necrosis. With this decrease in mortality, it is probably only a matter of time before minimally invasive pancreatic necrosectomy becomes the gold standard treatment for surgical intervention in patients with complicated necrotising pancreatitis (Connor et al, 2003, 2003, 2004).

Current limitations with treatments

The major obstacle in the treatment of acute pancreatitis is the lack of therapies available which are directed at disease progression at the earliest stage after initiation. Invasive support and better intensive care therapy is directed at supporting the major body organs/systems during the acute episode of the disease process, with the hope the disease will run a self limiting course without progression to the severe necrotizing form. During this first week of the disease, many patients will die despite maximal medical support available in the intensive care setting. If the patient is to suffer a severe episode of the disease, but survives the initial period, then treatments are directed at complications of the disease, such as pancreatic necrosis, abscess formation etc., causing another surge in mortality and morbidity.

It is in this part of the disease process that the greater improvements in care have been made with new surgical techniques that are increasingly reducing inpatient mortality and morbidity. It is however, noticeably, that in the overall scheme of things, we do not have treatments available which make any difference to the disease course at the very early stages when therapies would be most useful. It is at this point, within the first few hours of the onset of symptoms and presentation to the Emergency departments, which the greatest benefit to the patient would ensue. If a patient could be prevented from developing a severe course of the disease process, and experience a self limiting episode with no significant morbidity apparent, then the great burden both in human and financial terms of the disease process could be avoided.

Acute to Chronic Pancreatitis

Chronic pancreatitis is now considered to result from several episodes of acute pancreatitis, whether clinical or subclinical and the pancreatic damage increases as a continuum so long as the noxious stimuli are still present (Pandol et al, 1999). The majority of cases of chronic pancreatitis are secondary to alcohol, although patients with autoimmune and recurrent idiopathic episodes of acute pancreatitis are more susceptible. It is less common to witness chronic pancreatitis resulting from a small number of acute events related to other aetiologies such as gallstones. With the development of chronic pancreatitis, the risk of carcinogenesis increases dramatically and with it the development of pancreatic cancer (Go et al, 2005).

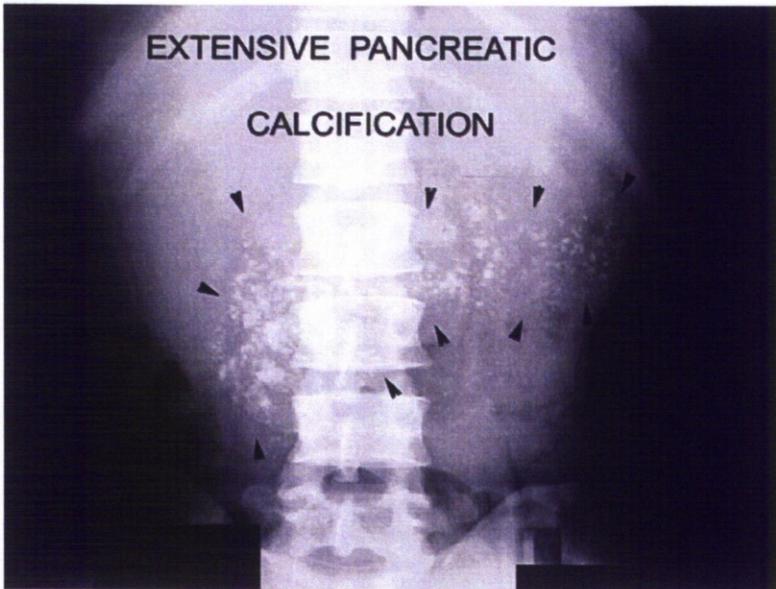
The evidence supports the recruitment of pancreatic stellate cells to be vital in the transformation of normal pancreatic tissue to fibrotic tissue pathognomonic of chronic pancreatitis. Stellate cells are thought to be generated in response to reactive oxygen species and cytokines, which as described previously, is part of the inflammatory cascade produced in response to premature trypsinogen activation (Vonlaufen et al, 2007).

In the normal pancreas, the stellate cells are in their quiescent state and located around the base of the acinus and store large amounts of vitamin A as lipid droplets. During pancreatic injury the cells are activated by a reduction of the vitamin A, leading to a fibroblast phenotype and the production of large amounts of extracellular matrix proteins such as collagen, fibronectin and laminin. With continuous pancreatic injury, the patchy areas of fibrosis eventually lead to clinically apparent chronic pancreatitis (Pandol et al, 2005, Petersen et al, 2006) (figure 1.6).

The major health costs of chronic pancreatitis relate to continued hospital admissions due to pain, treatment of disease such as diabetes mellitus and chronic malnutrition as well as an increased lifetime risk of developing pancreatic cancer (Howes et al, 2002).

Figure 1.6

Chronic Pancreatitis demonstrated on plain abdominal radiograph showing extensive calcific deposits along the entire length of the pancreas



Pancreatic resection demonstrate calcium deposit along the main pancreatic duct



However, if we are to identify therapies which may reduce the severity of acute pancreatitis, we must understand the earliest key pathological mechanisms so that interventions can be made at this point and so prevent the complications as described above. It is well established that the main functions of the pancreas are exocrine and endocrine, but it is the disruption in exocrine function that starts the inflammatory process in acute pancreatitis. The physiological processes in the stimulation of the exocrine pancreas have been described for over a century and involve neurohumoral mechanisms as described by Pavlov (1902), Bayliss (1902) and Harper and Raper (1943).

It is the pancreatic acinar cell which is responsible for exocrine function and, more specifically, production of proteases, which have the potential to destroy the pancreas. There are, however, important protective functions in place to prevent this from happening (Raraty et al 2000, Sutton et al, 2003). The main, of which, is that the proteases are produced by exocytosis, packaged in their inactive form, known as zymogen granules, to be activated when appropriate in the duodenum. There is now overwhelming evidence that premature activation of trypsinogen in the apical region of the pancreatic acinar cell is key to the autodigestive process, resulting in the inflammatory cascade of events culminating in acute pancreatitis (Raraty et al 2000, Sutton et al, 2003). Over the last decade disordered cytosolic Ca^{2+} signalling has been identified as playing a central role in the inappropriate intracellular enzyme activation (Ward et al, 1995, Raraty et al, 2000, Kruger et al, 2000) of trypsinogen early in the disease process (Leach et al, 1991) which leads to cytoskeleton disruption, co localisation of zymogens and lysosomes, vacuole formation and cytokine expression,

with resulting auto digestion and progression to clinical acute pancreatitis and in severe cases pancreatic necrosis, with the associated systemic sequelae (Gorelick et al, 1995, Ward et al, 1996, Neiderau et al 1990) (see figures 1.1-1.3).

However, there are still many pathological processes that remain unclear, for example, whether co localisation of lysosomes and zymogens plays a protective role in the process (Cancela et al, 2002). Therefore, it is obvious that much work is needed to be done to understand and solve the debates that rage between authors about what the exact pathophysiological events are, and only then can effective therapeutic targets be identified.

Normal anatomy and physiology of the Pancreas

To understand the pathophysiology of acute pancreatitis we must first determine what is considered normal. The pancreas was discovered by Herophilus (335 – 280 BC), a Greek anatomist and surgeon, and named a few hundred years later by Ruphus, also a Greek anatomist. The name 'Pancreas' comes from the Greek term *pan-kreas meaning* "all-meat", referring to its fleshy appearance.

Development and anatomy

The pancreas is a lobulated organ located in the retroperitoneum of the upper abdominal cavity behind the stomach. It develops from dorsal and ventral pancreatic buds of endodermal cells arising from the caudal part of the foregut. From week's five to seven of foetal development, the duodenum rotates to the right bringing the ventral and dorsal buds into contact with each other, where they fuse and the pancreatic ducts anastomose. The foetal pancreas begins to secrete insulin at ten weeks (Moore et al, 1993).

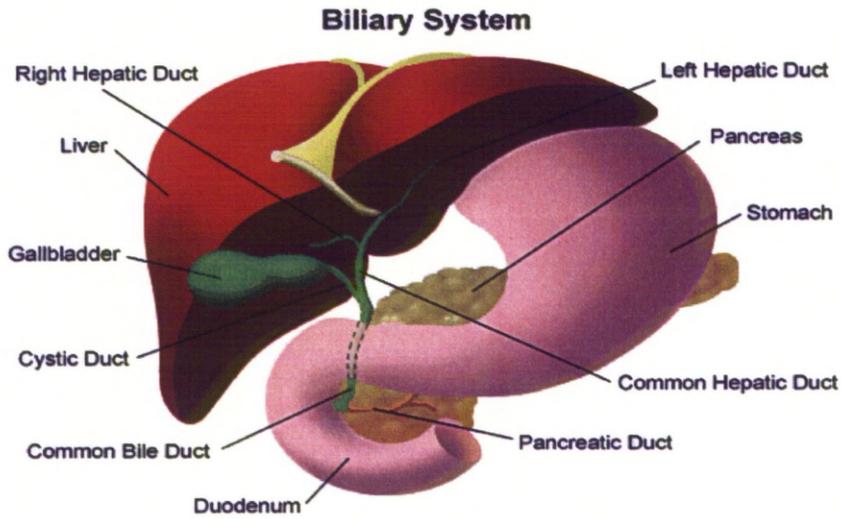
The pancreas eventually comes to lie in the epigastrium and left upper quadrant of the abdominal cavity. It is soft and situated on the posterior abdominal wall crossing the transpyloric plane. The gland is divided into four regions: the head, neck, body and tail (Kern, H.F. 1993).

The head of the pancreas is disc shaped and lies within the concavity of the duodenum (figure 1.7). A part of the head extends to the left behind the superior mesenteric vessels, called the uncinate process. The neck is the constricted portion of the pancreas

and connects the head to the body of the gland. It lies in front of the beginning of the portal vein and the origin of the superior mesenteric artery from the anterior aspect of the aorta (figure 1.7). The body runs in an upward direction and to the left across the midline and is somewhat triangular in cross section. The distal tail passes forward in the lienorenal ligament and comes into contact with the hilum of the spleen (Snell R, 1994). The large head is in close proximity to the duodenum and this is where the pancreatic duct(s) and common bile duct usually terminate at the major duodenal papilla (Ampulla of Vater) in the second part of the duodenum. It is the pancreatic ducts that transport the major pancreatic secretions into the duodenum to aid food digestion (figure 1.7). The main duct of the pancreas begins in the tail and runs the length of the gland, receiving numerous tributaries on the way. The accessory duct of the pancreas, when present, drains the upper part of the head and then opens into the duodenum a short distance above the main duct on the minor duodenal papilla. The accessory duct frequently communicates with the main duct. The pancreas receives its blood supply from the splenic and superior and inferior pancreaticoduodenal arteries, with the corresponding veins draining the pancreas into the portal vein. The lymph drainage is into lymph nodes situated along the arteries that supply the gland and ultimately drain into the coeliac and superior mesenteric lymph nodes. The nerve supply is from sympathetic and parasympathetic (Vagus nerve) nerve fibres (Snell R, 1994). The pancreas contains distinct exocrine, endocrine and ductal cells. It is the exocrine cells which form the large majority of the pancreas substance (around 98%) (Gorelick and Jamieson, 1994) and gives it its distinctive lobular appearance. Within the lobules, the acinar cell units are found which drain secreted products into intercalated and

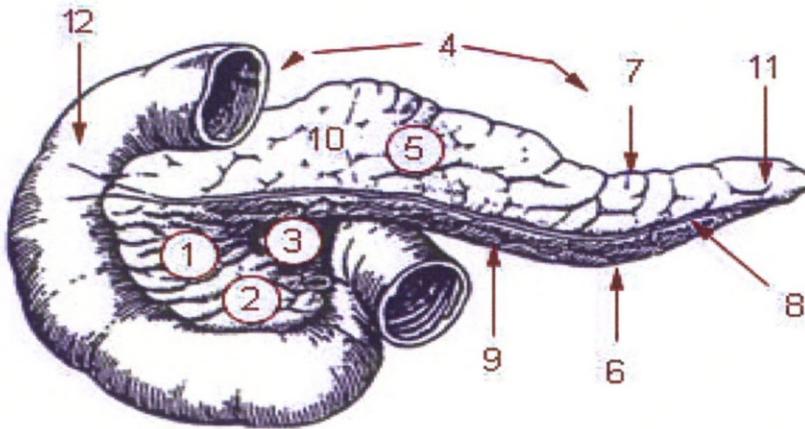
subsequently interlobular ducts, eventually draining into the main pancreatic ducts (figure 1.7). Endocrine cells or 'islets' are also found within the lobules. Each acinar unit contains several pancreatic acinar cells, which are highly polarised cells clustered around the duct with the apical pole full of zymogen granules ready for exocytosis into the duct when appropriate stimulation is applied.

Figure 1.7 (a)



Gross anatomy of the hepato-pancreato-biliary system. The pancreas lies posterior to the stomach with the head in contact with the duodenum, and the tail extends into the hilum of the spleen.

Figure 1.7 (b)



The pancreas with the stomach, and parts of the duodenum and biliary system cut away: 1) Head of the pancreas

- 2) Uncinate process
- 3) Pancreatic notch
- 4) Body of pancreas
- 5) Anterior surface
- 6) Inferior surface
- 7) Superior margin
- 8) Anterior margin
- 9) Inferior margin
- 10) Omental tuber
- 11) Tail of pancreas
- 12) Duodenum

Pancreatic Function

Pancreatic Endocrine Function

The endocrine portion of the gland, the islets of Langerhans (figure 1.8), which constitute only 2% of the mass of the pancreas are richly vascularized, allowing their secreted hormones (glucagon, insulin, somatostatin) ready access to the circulation. (Wharton, G.K et al., 1932; Henderson, J.R. 1969; Lifson, N. et al., 1980; Lifson, N. et al., 1985) Although islets comprise only 2% of the mass of the pancreas, they receive about 10 to 15% of the pancreatic blood flow. Additionally, they are innervated by parasympathetic and sympathetic neurons, and nerve signals clearly modulate secretion of insulin and glucagon (Daniel, P.M. et al, 1967).

The islets produce the hormones, principally insulin which is manufactured in the β cells, and glucagon from the α cells. The principle role of the hormones is control of carbohydrate metabolism, and therefore regulation of circulating blood sugar concentrations. Damage to this part of the pancreas results in disease processes such as diabetes mellitus (Korc, 1993). Insulin is a small protein (5800 kDa) consisting of two peptide chains called A and B, linked by two disulphide bonds. The A chain contains 21 amino acid residues and the B chain 30 residues. Beef and pig insulin differ from human insulin in only a few residues and are used in the treatment of diabetes. Human insulin is also generated by chemical manipulation of porcine insulin and by DNA recombinant technology. Insulin is synthesized as a larger single polypeptide preproinsulin, which is cleaved soon after synthesis to form proinsulin. Proinsulin is packaged into vesicles and converted to insulin by cleavage of a connecting peptide to form the two peptide chains

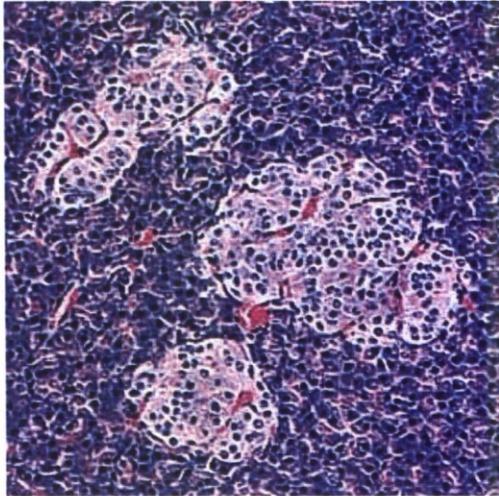
(Bray et al, 1994). Insulin is released from the cell by exocytosis in response to an increase in blood glucose and is secreted into the hepatic portal vein and thus reaches the liver directly. The active half life of insulin in blood is only a few minutes.

Insulin lowers blood glucose by facilitating glucose uptake in muscle and adipose tissue and by inhibiting hepatic glucose output. The most important effects of insulin are in the liver, where it stimulates glycogen and fat synthesis and inhibits glycogen breakdown and ketone body formation. In muscle it stimulates glucose and amino acid uptake, and glycogen and protein synthesis. In adipose tissue it stimulates glucose uptake and triglyceride synthesis. It also increases potassium uptake into cells and can consequently lower plasma potassium levels which is used in the potentially fatal clinical situation of hyperkalaemia (Bray et al, 1994).

Glucagon is a peptide (3500 kDa) which is secreted by the pancreas in response to low blood sugar and, in contrast to insulin, it increases blood glucose levels. Glucagon acts on the liver to stimulate glycogenolysis (breakdown of glycogen) and gluconeogenesis (synthesis of glucose from lactate, amino acids or glycerol). Glucagon secretion is also stimulated by amino acids, as is insulin release. This ensures that, when there is a high intake of amino acids, a precipitous fall in blood glucose, due to elevated insulin, is prevented by the action of glucagon. Glucagon is also a potent stimulator of fat mobilization (Bray et al, 1994).

Figure 1.8

Histological section of the endocrine portion of the pancreas



The endocrine portion of the pancreas takes the form of many small clusters of cells called islets of Langerhans or, more simply, islets. Humans have roughly one million islets. In standard histological sections of the pancreas, islets are seen as relatively pale-staining groups of cells embedded in a sea of darker-staining exocrine tissue. The image above shows three islets (pale stain) in the pancreas of a horse.

Pancreatic Exocrine Function

The focus of this study, however, is the exocrine pancreas, and therefore further description of the pancreas physiology and pathophysiology will be limited to this aspect of the gland.

The pancreas secretes about 1.5 litres of fluid per day. The pancreatic secretions which drain into the duodenum contain enzymes which digest fats, proteins, polysaccharides, DNA and RNA. High levels of bicarbonate and water are also found in the pancreatic juice giving a relatively high pH when it enters the duodenum and contributes to the neutralisation of the acid chyme which enters the duodenum from the stomach. It is the ductal cells which are responsible for bicarbonate and water secretion, whereas the acinar cells secrete the inactive enzymes or zymogens. The rate of secretion is under the control of nerves and hormones (Gorelick and Jamieson, 1994).

The neural stimulation comes from cholinergic and peptidergic sources with the major stimulants being acetylcholine (ACh), vasoactive intestinal peptide (VIP), neuropeptide Y and gastrin-releasing peptide (GRP) (Holst, 1993). Hormonal control is mediated by Cholecystikinin, secreted into the blood stream by the duodenal I cells (Chey, 1993) and is responsible for acinar cell stimulation and secretin which predominantly acts on ductal cells to stimulate bicarbonate and water production (Case et al, 1993, Steer et al 2004).

A cephalic phase of pancreatic secretion is recognised in which enzyme rich fluid is stimulated by vagal activity. This early phase of secretion is supplemented during the gastric phase of digestion by a stretch –activated vagally mediated reflex and release of gastrin by the G cells situated in the antrum of the stomach. The major control is,

however, exerted during the intestinal phase when nerves, and more importantly, the intestinal hormones determine pancreatic control. The entry of the chyme into the duodenum is generally followed by the secretion of a bicarbonate and enzyme rich fluid from the pancreas. The hormones mostly responsible are secretin (ductal cell stimulation) and cholecystokinin (acinar cell stimulation). Secretion is also inhibited by several peptides including somatostatin – analogues of which are used clinically to reduce pancreatic secretions, pancreatic polypeptide and glucagon (inhibits duct secretion).

Functions of pancreatic secretions

The bicarbonate rich secretion of the pancreas and also, to an extent, the liver contribute to the neutralisation of the acid chyme. Thus a favourable pH is established for the pancreatic enzymes, which have optimal activities in the 6.7 – 9.0 pH range.

The pancreatic enzymes or their precursors are secreted along with the aqueous secretion and include:

- 1) protease precursors – trypsinogen , chymotrypsinogen and procarboxypeptidases – which are secreted by the pancreatic acinar cells and activated in the small intestine. The activation is initiated by the conversion of trypsinogen to trypsin by the intestinal enzymes, enterokinase, found in the apical membrane of the intestinal epithelial cells. The trypsin in turn activates trypsinogen autocatalytically and also activates the other precursors;

- 2) an active α - amylase which splits α -1,4-glycosidic bonds and hydrolyses starch to mainly maltose;
- 3) lipases which act on triglycerides and phospholipids;
- 4) a number of other enzymes, eg. Ribonuclease, elastase and collagenase that act on specific macromolecules.

The pancreatic acinar cell

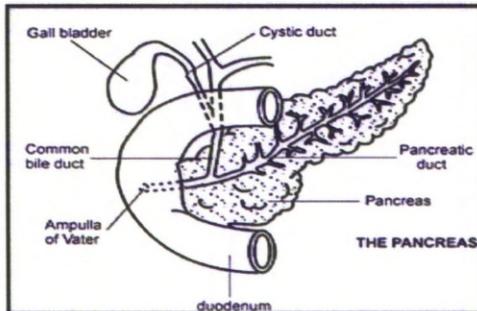
It is the pancreatic acinar cells which are responsible for producing and secreting enzymes and also fluid in the form of water. The cells retain many functional characteristics when isolated from the gland, and therefore have been the subject of many studies examining normal physiological and pathophysiological processes.

In the organ, and when isolated as cell clusters, the acinar cells are linked together by tight junctions and form the acinar cell units, the functional structures of pancreatic enzyme secretion (figures 1.9 and 1.10). Zymogen granules are located at the apical pole and contain the enzymatic component of the pancreatic secretion (figure 1.11). The endoplasmic reticulum (ER) is densely packed throughout the basolateral region of the cell which projects into the apical region through strand-like projections (Park et al, 2000). The nucleus is always in the basal region of the cell (Bolender, 1974). There are three main areas in the cell where the mitochondria congregate – the nuclear region, sub – plasma membrane and most predominantly, in the perigranular portion around the zymogen granules of the cell (Tinel et al, 1999). The golgi apparatus is formed in a discrete layer between the ER and the perigranular mitochondria (Dolman et al, 2006).

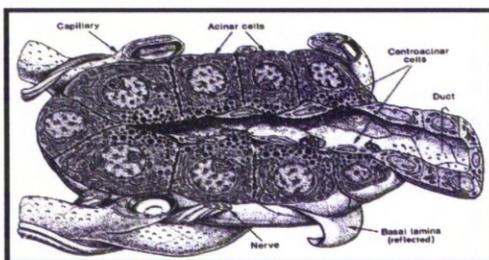
The zymogen granules are exocytosed across the luminal membrane in response to stimulation with the secretagogues ACh, CCK, VIP and GRH (bombesin). The exocytotic secretion is accompanied by the expulsion of NaCl rich fluid into the lumen which facilitates the flow of enzymes into the main pancreatic ducts.

Figure 1.9: Anatomy of the exocrine pancreas

A)



B)

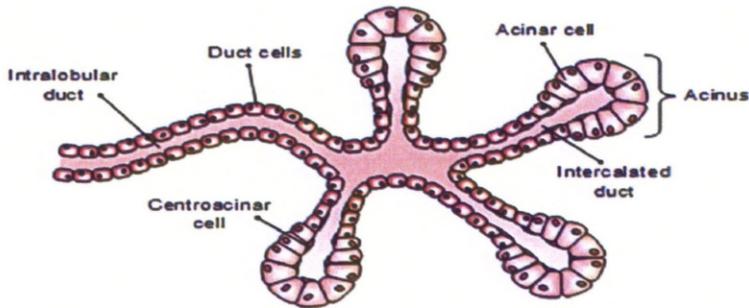


(A) The location and structure of the pancreas, note the pancreatic duct drains exocrine secretions from all parts of the pancreas into the duodenum.

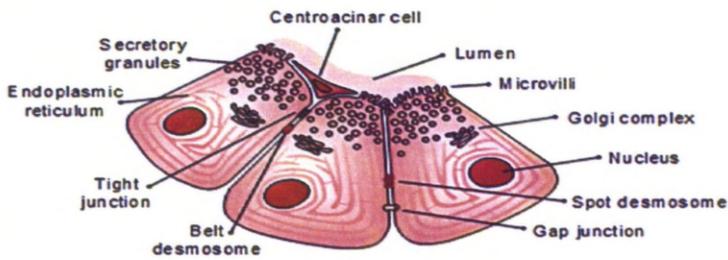
(B) Schematic diagram of the structure of the pancreatic acinar unit, the acinar cells form a tight epithelial sheet around a lumen into which secretion takes place across the apical membrane of the cells. The lumen drains into the pancreatic duct. There is strong structural polarity in the cells with the zymogen granules exclusively located in the apical region and the nucleus found in the basal region (Motta et al, 1997)

Figure 1.10

**The basic structure of the adult exocrine pancreas:
Structure of pancreatic acini**

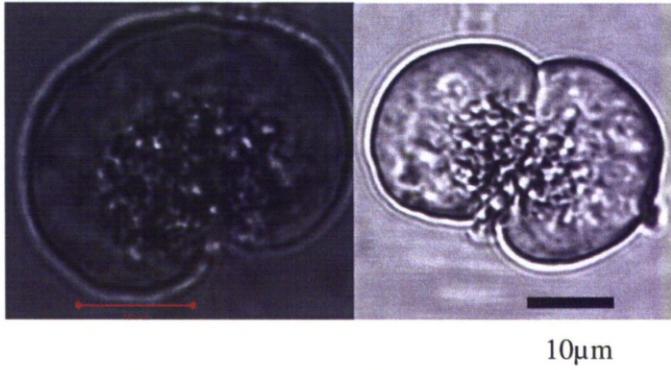


**The basic structure of the adult exocrine pancreas:
Structure of pancreatic acini**

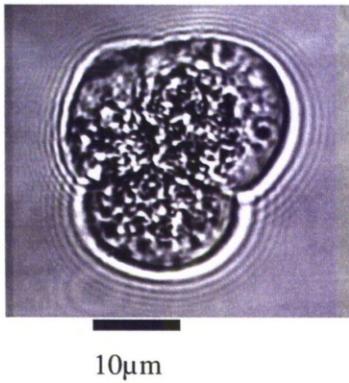


Schematic representation of a pancreatic acinus, demonstrating cells surrounding a duct. The polarised apical portion is in close proximity to the duct lumen.

Figure 1.11(a)



(b)



(a) Transmitted light image of isolated murine pancreatic acinar cells (top insets), note that the polarity of the cells is maintained with the dark zymogen granules clustered at the apical poles of each cell.

(b) Transmitted light images of isolated human pancreatic acinar cells (below –triplet) which maintain remarkable similarity to the murine form with granules tightly packed at the apical poles.

Cellular physiology in the health and disease

Normal intracellular signalling in the pancreatic acinar cell

The Ca^{2+} ion is a simple, evolutionarily ancient and universal second messenger with a variety of physiological effects throughout the animal kingdom. These actions include the control of cell growth, initiation of muscle contraction, platelet activation, control of secretion, gene expression and the triggering of apoptosis (Raraty et al 1999, Ashby 2002).

The acinar cell function is principally the production of zymogens that can be cleaved in the duodenum. The process in zymogen production can be broken down into six different distinct steps: synthesis, segregation, intracellular transport, concentration, storage and discharge (Palade 1975). It is the final stage of this process that is thought to be principally disrupted in the initiation of acute pancreatitis.

The term 'stimulus-secretion coupling' was first described in the 1960's (Douglas et al, 1961) and describes the process whereby a stimulant applied to a cell triggers a chain of events that ultimately produces the secretory response. Later it was postulated that exocytosis of the zymogen was controlled by intracellular Ca^{2+} (Douglas et al, 1968).

The acinar cell shows strong structural polarity with the zymogen granules being concentrated at the apical pole of the cell and the nucleus situated towards the base of the cell surrounded by rough endoplasmic reticulum (figure 1.11, 1.12) which occupies most of the basolateral region of the cell as an extensive connected tubular system. It is of interest that under 'normal' resting conditions: the concentration of calcium within the

cytosol of the acinar cell is in the region of 10^{-7} M, compared with 10^{-3} M in the extracellular fluid, and 10^{-4} M in the internal stores, - mostly the endoplasmic reticulum (Sutton et al, 2003). The importance of this is that the differences in the concentrations favour Ca^{2+} entry into the cell down a concentration gradient. The concentration gradient is maintained by a magnesium dependent Ca^{2+} ATPase plasma membrane pump (PMCA) and by an endoplasmic reticulum Ca^{2+} ATPase pump (SERCA pump). There are also a number of specific Ca^{2+} entry channels, which are controlled by agonists and by changes in the filling status of the intracellular stores. The maintenance of a low Ca^{2+} concentration within the cytosol enables small local increases of Ca^{2+} in different regions of the cell to be utilised as a signal to control intracellular events (Raraty et al, 1999, Sutton et al, 2003).

Ca^{2+} oscillations are the major form of intracellular signalling in secretory epithelial cells, stimulated by neurotransmitters and hormones (figure 1.12) (Ashby et al, 2002, Berridge et al, 2000, Williams et al, 2001). A common trend is that the Ca^{2+} transients are initiated in the apical region of the cell (Ashby et al 2002). The cell surface receptors (muscarinic receptors) are located at the basolateral membrane and there is intracellular long distance communication leading to Ca^{2+} release in the apical region of the cell (Ashby et al, 2003).

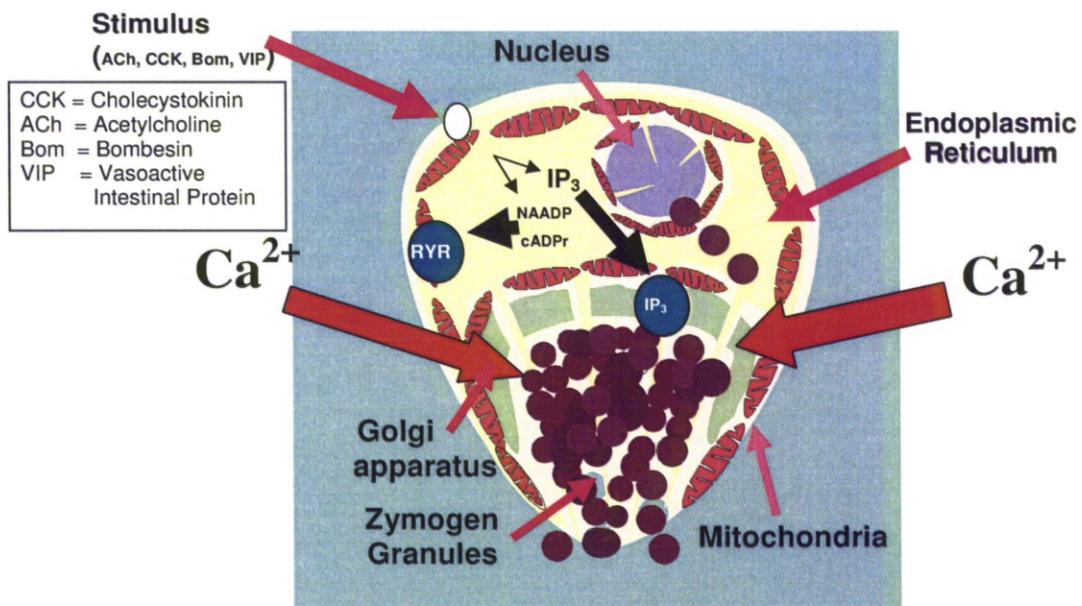
The pathway begins with binding of extracellular messenger molecules to G- protein linked transmembrane receptors (Ward et al, 1995). The exocytic stimulants used in the majority of animal pancreatic acinar cell experimental models are the neurotransmitter, Acetylcholine (ACh), and the hormone Cholecystokinin (CCK) or an analogue, Cerulein. When presented to the cell surface membrane, conformational change occurs in the G

– protein linked receptors, which, in the case of ACh, stimulate muscarinic receptors activating isositide-specific phospholipase C. This hydrolyses phosphatidylinositol-4, 5-bisphosphate to inositol -1, 4, 5- triphosphate (IP₃) and diacylglycerol (Williams et al, 2001). CCK causes conformational change in G – protein receptors generating cyclic ADP ribose and nicotinic acid adenine diphosphate (NAADP) (see figure 1.13). The product of which, like ACh, is dependent on IP₃, causing release, via specific IP₃ receptors on the endoplasmic reticulum (ER) membrane, of Ca²⁺ from the ER terminals within the apical, granular pole of the cell (Williams et al, 2001, Ashby et al, 2002). Unlike ACh, cADPr and NAADP also causes Ca²⁺ release via the ryanodine receptor located throughout the acinar cell cytosol (Cancela et al, 2000, Hussain et al, 2005). However, it is still unclear as to whether NAADP has a separate Ca²⁺ release channel or acts at ryanodine receptor, but it is the most potent Ca²⁺ releasing second messenger known (Gallione et al, 2005).

Figure 1.12

Normal Ca^{2+} signalling in pancreatic acinar cells

Schematic representation of the acinar cell showing cellular polarity with the zymogen granules concentrated at the apical pole of the cell. Note the large area (yellow) occupied by the endoplasmic reticulum, which is the major intracellular store of calcium. The cell has various receptors on the basal membrane responsible for the neurohumoral stimulated Ca^{2+} release via second messengers with subsequent zymogen exocytosis.



With low threshold concentrations of ACh or CCK, repetitive local cytosolic Ca^{2+} oscillations are generated (figure 1.13) in the apical part of the cell associated with concurrent exocytosis of zymogen granules through the apical plasma membrane (Muryama et al, 1993) and opening of Ca^{2+} dependent chloride (Cl^-) channels present exclusively in the apical plasma membrane, which regulates acinar cell fluid secretion (Park et al, 2001).

Cerulein or CCK causes Ca^{2+} release dependent on functional NAADP and cyclic ADP - ribose as well as IP_3 receptors which dramatically potentiate the response in the apical pole of the cell and can initiate a global response. ACh causes global response via NAADP or cADP receptors but not via IP_3 receptors. From this we can deduce that two different intracellular pathways converge on a common oscillator pathway (Cancela et al, 2000) (see figure 1.14). The global response initiated by higher concentrations of ACh or CCK, travels across the cell by Ca^{2+} induced Ca^{2+} release (CICR) (Ashby et al 2003). This phenomenon is initiated in the highly sensitive apical pole of the cell where there are both IP_3 and ryanodine receptors present. The Ca^{2+} wave is carried to the basal part of the cell where regeneration can take place. The buffering capacity of the pancreatic acinar cell is very high, with a buffering capacity of around 2000 (i.e. if 2000 calcium ions were added to the cell, only 1 would remain free and the rest bound by buffer) which indicates that localised apical signalling must be overwhelmed to allow propagation of Ca^{2+} waves (Mogami et al, 1999). It is interesting that the CICR cannot be generated in the basal part of the cell, as it is particularly insensitive in this region and is advantageous with regards to maintaining the polarity of the cell.

As the acinar cell is highly polarised, it is suited to its purpose of zymogen production, and the majority of the Ca^{2+} signalling takes place in the apical pole of the cell. Following release of Ca^{2+} from the endoplasmic reticulum, the normal oscillatory signal is usually prevented from spreading to the basolateral region of the cell by a high concentration of mitochondria, which form a perigranular belt and provide the buffer barrier. It is also believed that the concentration of mitochondria is high in this region of the cell as this is where there is high metabolic demand (Park et al, 2001). Therefore, at the sites where the mitochondria are in close proximity to the endoplasmic reticulum, micro domains of high Ca^{2+} concentrations are generated by Ca^{2+} release from endoplasmic reticulum through IP_3 receptors. These high local Ca^{2+} concentrations trigger mitochondrial Ca^{2+} accumulation and therefore mitochondrial Ca^{2+} concentration rises accordingly. The result of which, stimulate activity of dehydrogenases of the Krebs cycle and modulates ATP production.

The concentration of Ca^{2+} in the apical pole is prevented from becoming toxic by extrusion of Ca^{2+} by Ca^{2+} ATPase pumps at high concentrations by the apical plasma membrane and also by exocytosis (Sutton et al, 2003, Petersen, 2005, Park et al 2001). The small Ca^{2+} fluxes initiate re-entry of small quantities of Ca^{2+} through store operated channels in the basal membrane driven by endoplasmic reticulum uptake via Ca^{2+} ATPase and through endocytosis. With this mechanism in place, the ER is not devoid of its Ca^{2+} stores too quickly during stimulation and can go on for some time even in the absence of an external store of Ca^{2+} (Petersen, 2005).

In the event of supramaximal stimulation the endoplasmic reticulum Ca^{2+} stores are depleted and the cytosolic concentration of Ca^{2+} increases (Petersen 2004), which, if

the stimulus be removed, the basal cytosolic concentrations of Ca^{2+} are restored within seconds. The complete refilling of the Ca^{2+} stores in the ER takes several minutes and is mediated by the ER Ca^{2+} pump without any elevation in the bulk cytosolic Ca^{2+} concentration. As the ER Ca^{2+} concentration increases, the rate of Ca^{2+} uptake declines until finally it reaches equilibrium with the passive leak rate, previously described (Camello et al, 2002), and establishes the normal high ER concentration. As well as the perigranular mitochondria, the peripheral mitochondria positioned near the nucleus delay or prevent Ca^{2+} entry into the nucleoplasm (Tinel et al, 1999). High concentrations of mitochondria can also be found situated next to the basolateral membrane and take up Ca^{2+} during store-operated entry. All groups of mitochondria are positioned in such a way that is suited to specific subcellular demands (Tinel et al, 1999).

If supramaximal stimulation occurs persistently, or when all three second messengers (IP_3 , NAADP and cADP ribose), are administered together even at threshold concentrations, a dramatic potential results in sustained, global, cytosolic Ca^{2+} elevation (Cancela et al, 2002, Cancela et al, 2002) (see figure 1.14). This response is observed with hyperstimulation, ductal hypertension, bile salts and non-oxidative metabolites of alcohol - fatty acid ethyl esters (Voronina et al, 2002, Criddle et al 2004, Criddle et al, 2006). This disruption in cell signalling is thought to lead to inappropriate enzyme activation, the hallmark of acute pancreatitis (Sutton et al, 2003, Kruger et al, 2000, Raraty et al, 2000, Raraty et al, 2005, Raraty et al, 2002).

Figure 1.13

Normal physiological Ca^{2+} signalling in response to ACh (top figure) and CCK (bottom figure) in murine pancreatic acinar cells

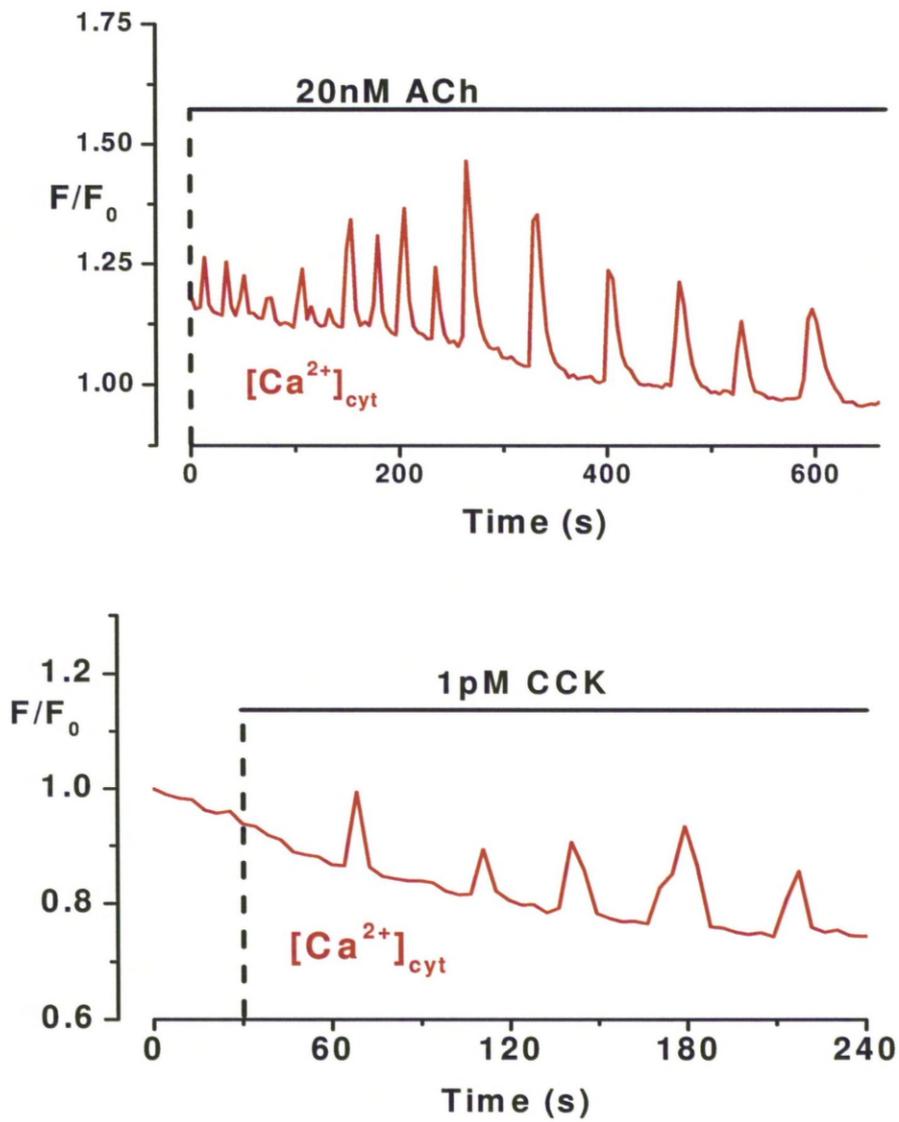


Figure 1.14 Secretagogue stimulation (Ach and CCK) generates Ca^{2+} signals via two different intracellular pathways that converge on a common oscillator pathway

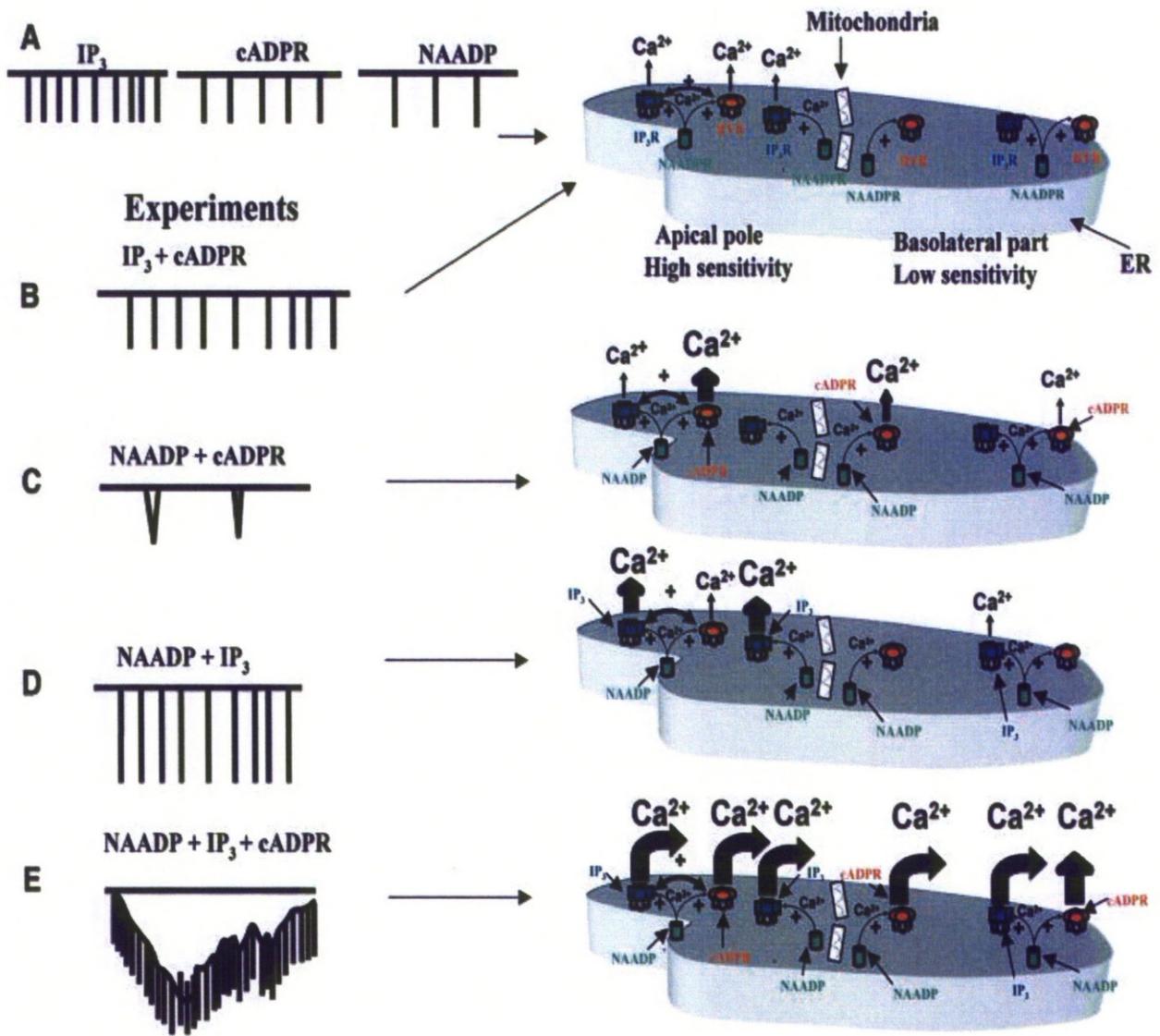


Figure 1.14

(A) Schematic model showing that every messenger on its own can initiate local Ca^{2+} spikes.

(B) There is little interaction between IP_3 and cADPR.

(C) NAADP does potentiate the action of cADPR producing long-lasting global spikes at long intervals.

(D) In contrast, NAADP only has a locally potentiating effect on the local IP_3 -evoked Ca^{2+} spikes.

(E) When all three messengers act together a large, sustained, global Ca^{2+} elevation is observed. The apical pole is the most sensitive part of the cell. In the models shown to the right, the basolateral part of the cell contains poorly sensitive Ca^{2+} release units that cannot trigger a wave in the presence of either IP_3 , cADPR or NAADP alone. To generate a Ca^{2+} wave across the cell, a combination of potentiated Ca^{2+} release in the apical pole, helping to overcome the mitochondrial barrier and sensitization of Ca^{2+} release channels by coincident activation of ryanodine, IP_3 and NAADP receptors by their respective messengers in the basal pole is necessary.

From Cancela et al, 2002

Premature intracellular cleavage of trypsinogen - the hallmark of acute pancreatitis

The autolytic process in acute pancreatitis is precipitated by activated enzymes which are found both within the apical granular area of the acinar cell and around the cell early in the disease process (Mithofer et al, 1998, Neiderau et al, 1990, Hofbauer et al, 1998). We know that premature activation of trypsinogen occurs consistently with all known precipitating factors in pancreatitis and therefore it is believed that trypsinogen cleavage in the acinar cell is the 'master switch' leading to the clinical features of pancreatitis (Whitcomb et al, 1996, Sutton et al 2003). There are, however, many other pro – enzymes that have differing significance. Recent evidence suggests that granular proteases, pro-elastase and pro-cathepsin B, may also have important roles to play in acinar cell damage (Mayerle et al, 2005, Ruthenburger et al, 2006).

Despite the series of safeguards that are present to prevent the inappropriate activation of trypsinogen, there is a small group of patients who are particularly susceptible to the disease as a result of a genetic defect in the gene coding for trypsinogen production. As trypsinogen is thought of the gateway to the onset of acute pancreatitis, patients with the genetic PRSS1 cationic trypsinogen mutation, (including R122H and N29I), render prematurely activated intracellular trypsin resistant to normal, protective inactivation and subsequent recurrent pancreatitis results (Sahin-Toth et al, 2001, Howes et al, 2005). This process subjects the patient to a 40-50% lifetime risk of developing pancreatic cancer (Vitone et al, 2006, Howes et al 2002, 2005).

Pathogenesis of acute pancreatitis

Supramaximal stimulation of the acinar G-protein linked receptors produces morphological, biochemical and physiological similarities with the human form of acute pancreatitis (Raraty et al, 1999, 2000, Ward et al 1995, 1996, Sutton et al, 2003).

Following caerulein induced pancreatitis in vivo, trypsinogen activation and vacuole formation were seen in the apical pole of the acinus in vitro. The vacuoles are thought to result from fusion of zymogens and lysosomes (Kruger et al, 2000, Raraty et al, 2000, Sutton et al, 2003). This effect is also observed when the acinar cells are treated with thapsigargin, an endoplasmic reticulum Ca^{2+} ATPase antagonist, preventing reuptake of Ca^{2+} from the cytosol back into the endoplasmic reticulum. Both of the treatments produce a sustained rise in cytosolic Ca^{2+} with subsequent trypsinogen activation and vacuole formation (Ward et al, 1995). More recently, using fluorescent dyes coupled to enzyme – specific amino acid moieties and visualisation by confocal microscope, within 300 seconds of hyperstimulation, fluorescence was observed within multiple, discrete rounded compartments, each up to 1 micrometer diameter and situated throughout the granular region of the cell indicating trypsinogen activation. By 60 minutes, typical vacuoles were demonstrable by electron micrograph (Raraty et al, 2000) (figure 1.15).

This response has, experimentally, been prevented by addition of the membrane-permeable specific Ca^{2+} chelating agent BAPTA-AM, as well as simply removing extracellular Ca^{2+} (Raraty et al, 2000). With BAPTA-AM no lysosomal cathepsin B activation occurred, with any morphological abnormalities in the apical region of cells, which again, confirms dependence on abnormal Ca^{2+} signalling (Mogami et al, 1998, Cancela et al, 2002).

What is apparent from this work is the spatio-temporal characteristics of intracellular enzyme activation suggests activation occurs first within the zymogen granules themselves, and that activated granules then become vacuoles, which then leak the activated enzymes into the surrounding cytosol with subsequent damage (Raraty et al, 2002).

Figure 1.15

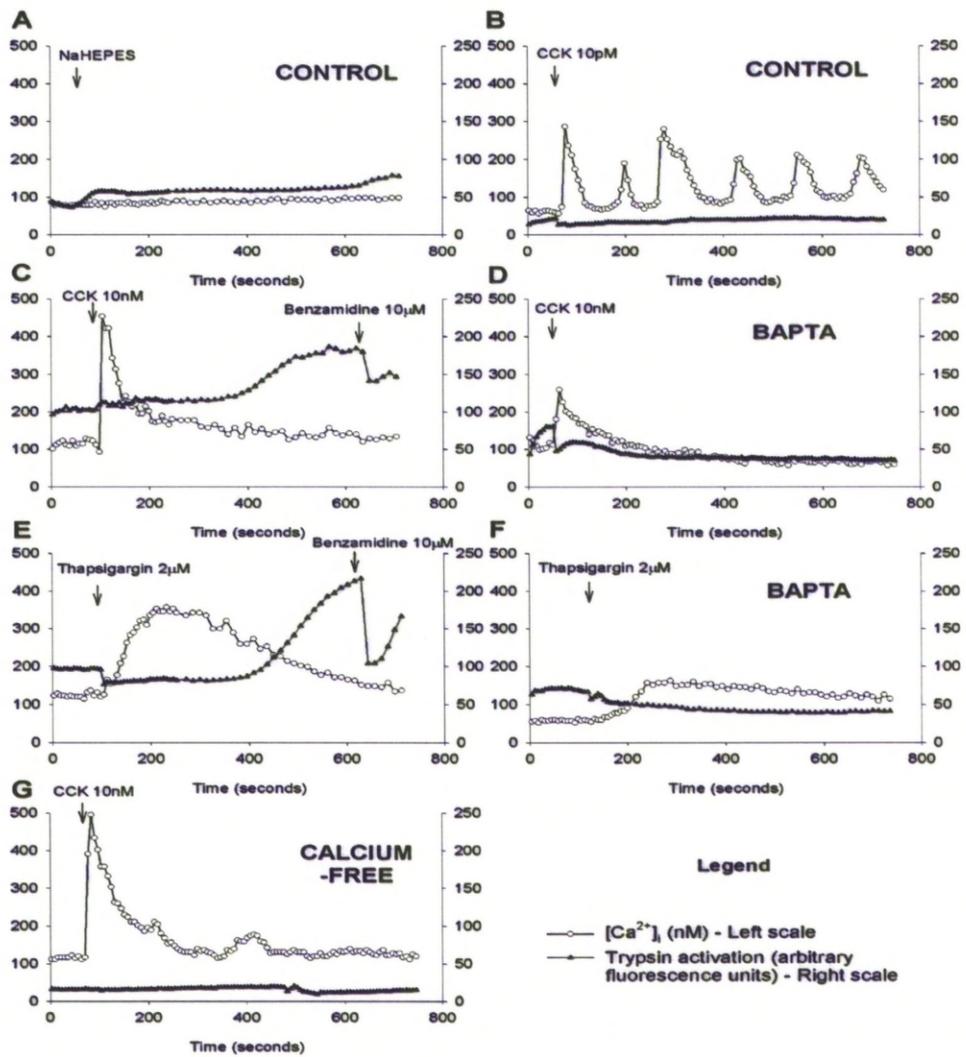


Figure 1.15

Simultaneous measurement of $[Ca^{2+}]_c$ response with fura-2 (nM, plotted on left scale) and trypsin activity with BZiPAR (arbitrary fluorescence units, plotted on right scale).

(A) Non-stimulated control. Little change in either $[Ca^{2+}]_c$ or BZiPAR fluorescence.

(B) Stimulation with 10 pM CCK elicits typical $[Ca^{2+}]_c$ oscillations without trypsin activation.

(C) Stimulation with 10 nM CCK evokes an immediate elevation of $[Ca^{2+}]_c$ followed by a prolonged plateau phase. After a delay of approximately 300 seconds, there is a rise in trypsin activity to an elevated plateau level. Addition of 10 μ M benzamidine, a cell-permeable trypsin inhibitor, reduces activity.

(D) Stimulation with 10 nM CCK after pretreatment with BAPTA produces an attenuated $[Ca^{2+}]_c$ response and no trypsin activation.

(E) Thapsigargin (2 μ M) evokes a broad $[Ca^{2+}]_c$ response, and there is marked enzyme activation after 300 seconds, which is subsequently reduced by 10 μ M benzamidine.

(F) After BAPTA preloading, thapsigargin elicits very little change in $[Ca^{2+}]_c$ and there is no sign of trypsin activity.

(G) In the absence of external Ca^{2+} , 10 nM CCK evokes a normal transient $[Ca^{2+}]_c$ rise but no plateau phase and also no enzyme activation.

From Raraty et al. 2000

Extracellular Ca²⁺ and Store Operated Channels

Hypercalcaemia is an important aetiology of acute pancreatitis in clinical practice, and therefore the effect of extracellular Ca²⁺ has been investigated in some detail. Before disordered Ca²⁺ signalling was hypothesised in 1995 (Ward et al, 1995) other theories as to why hypercalcaemia may induce pancreatitis were postulated and indeed, the earliest of which, supported the theory put forward by Opie. In this situation, the persistent hypercalcaemic precipitation of stones in the pancreatic duct causes ductal hypertension in a similar fashion as gallstones do, within a common channel (Goebell et al, 1976).

After this, other notions included blockage of secretory granule exocytosis and subsequent condensing vacuole formation in the granular area of the cell (Frick et al, 1992, 1994, 1995, 1991). The investigators observed that small protein precipitates were present in the cytosolic component of the cell when subjected to increased Ca²⁺ concentrations, which may disrupt the acinar cell cytoskeleton and further contribute to the disease process (Frick et al, 1990).

However, the importance of disordered Ca²⁺ signalling in the initiation of acute pancreatitis has provoked detailed investigation into the importance of extracellular Ca²⁺. In the absence of extracellular Ca²⁺ present in the external bathing solution, no significant sustained rise in the intracellular Ca²⁺ concentration results in response to supramaximal stimulation with no appreciable vacuole formation and no morphological signs of acute pancreatitis (Raraty et al, 2000). After the initial peak release in Ca²⁺ is observed a further elevation in Ca²⁺ persists with continuing hyperstimulation, which is dependent on the continued entry of Ca²⁺ from the external medium via store operated

channels. If the concentration of external Ca^{2+} is increased, the amount of trypsinogen activated is increased, with vacuole formation (Yoo et al, 2000).

CCK- 8 and CCK- 58 in experimental models

The physiological relevance of CCK 58 wasn't certain as it has been assumed that all actions of CCK on the receptor are mediated by its COOH-terminal seven amino acids and therefore, little point in questioning the relevance of CCK 8 in the experimental and clinical fields. Data gained a few years ago, however, tested the different isoforms of CCK that yielded interesting results and demonstrated that it is CCK 58 that is the most abundant form of CCK circulating in humans (Reeve et al, 2005). The data also suggests that CCK 58 stimulates fluid, as well as zymogen, secretion when presented to the G- protein linked receptors, conventionally thought to be produced by the duct cells in response to secretin stimulation. (Reeve et al, 2003, 2005, Yamamoto et al, 2005, Criddle et al, 2007).

Recent experimental data, however, has determined that CCK-8 and CCK-58 have essentially identical actions on the acinar cell at high and low agonist concentrations, suggesting an action via the same receptor and that the differences observed in an intact rat model may result from indirect effects of the peptides. The data strengthens the argument that CCK-58 is an important physiological form of the hormone to be used in future experimentation with both human and murine cells (Criddle et al, 2009).

Ethanol and gallstones in acute pancreatitis

Gallstones, ductal obstruction and bile acid reflux

Excessive ethanol ingestion and gallstone disease are the commonest aetiologies of acute pancreatitis in the Western world. How this relates to disordered Ca^{2+} signalling was unclear until experimental studies performed within the last decade or so (Raraty et al, 2000, Voronina et al, 2002, Criddle et al 2004, 2006).

Early animal experiment work suggested that gallstones result in acute pancreatitis via pure ductal obstruction and hypertension (O'Konski et al, 1990, Opie et al, 1901). Opie originally described a link between gallstones and pancreatitis in the early 20th century and led to the common channel hypothesis (Opie, 1901). In the majority of cases the symptoms are improved if not abolished by relief of ductal obstruction (Benifla et al, 2003, Neblett et al 2000, Kamisawa et al, 2003). Early experimental work also demonstrates this in which the pancreatic and biliary ducts were ligated and necrotizing haemorrhagic pancreatitis ensues, which does not progress with relief of obstruction (Lerch et al, 1992, 1993, Runzi et al, 1993).

However, recent evidence suggests the pathological insult delivered to the pancreatic acinar cell may actually be initiated by the resultant bile reflux which is in contact with the acinar cells, at higher concentrations than under normal physiological circumstances, and for prolonged periods of time when ductal obstruction is present secondary to common bile duct stones lodged at the Ampulla of Vater (Voronina et al, 2002).

The ability of bile acids to trigger acute pancreatitis has been confirmed in a number of studies (Niederau et al, 1990; Senninger, 1992), but the pathological cellular mechanisms of such bile-mediated injury were unclear.

The cellular mechanisms underlying the toxic bile acid effects have been studied in more detail in liver. In these studies it has been found that the highly toxic monohydroxy bile acids trigger calcium signals in hepatocytes (Anwer *et al.* 1988; Combettes *et al.* 1988) and it has been documented that the naturally occurring bile acids tauroolithocholate (TLC) and its sulfated form (TLC-S) release calcium from intracellular stores in hepatocytes (Combettes *et al.* 1988, 1990). TLC-S induced calcium release from the same store as IP₃, but the release mechanism was different (Combettes *et al.* 1990).

In the pancreas, Tauroolithocholic acid 3-sulfate (TLC-S) was demonstrated to be able to cross the acinar cell plasma membrane and induce Ca²⁺ oscillations localised to the secretory granule in the apical region of the cell at relatively low concentrations. Global Ca²⁺ signals were also produced originating in the apical part and spreading to the basal part of the cell with increasing concentrations of the bile acid (Voronina et al, 2002). The signals were unaffected by atropine (muscarinic receptor antagonist) but abolished by caffeine (IP₃ receptor antagonist) or by prior depletion of Ca²⁺ stores, suggesting that the Ca²⁺ releasing properties of bile acids via intracellular second messengers (IP₃R, cADPr, NAADP) could play an important role in the Ca²⁺ toxicity witnessed during experimental acute pancreatitis (Voronina, S et al, 2002, 2005, Gerasimenko, JV et al 2006) (figure 1.16). Further evidence demonstrated that bile acids can release Ca²⁺ from both the ER and an acidic store in the secretory granule

area. In both stores TLC-S interacts with both the IP₃Rs and the ryanodine receptors (RyRs). TLC-S opens RyRs through activation of the NAADP, but not the cADPR pathway (Gerasimenko, JV et al 2006), again demonstrating how bile acids can contribute to a possible toxic [Ca²⁺] rise.

Other bile acids were also examined such as Taurodeoxycholate (TDC) and Taurochenodeoxycholate (TCDC) and, although some [Ca²⁺] oscillations were observed, no pathological responses were noted (Voronina et al, 2002). The taurine-conjugated bile acids have also been shown to act as detergents, but the demonstration that the [Ca²⁺] rises were abolished by caffeine would argue against this being a major factor in generating toxic [Ca²⁺] signals.

It is the long term elevation of the cytosolic calcium concentration (calcium plateau), which depends on continued calcium influx from the external solution, has been shown to be a particularly dangerous signal, promoting activation of trypsinogen inside pancreatic acinar cells (Raraty *et al.* 2000; Kruger *et al.* 2000). TLC-S was shown to be capable of generating this form of calcium signal. Oscillatory patterns were more prominent at low doses of TLC-S, whilst formation of long-lasting calcium plateaus (potentially more damaging signals) were more common at high doses suggesting calcium toxicity as a possible mechanism for bile-induced injury to pancreatic acinar cells.

Figure 1.16

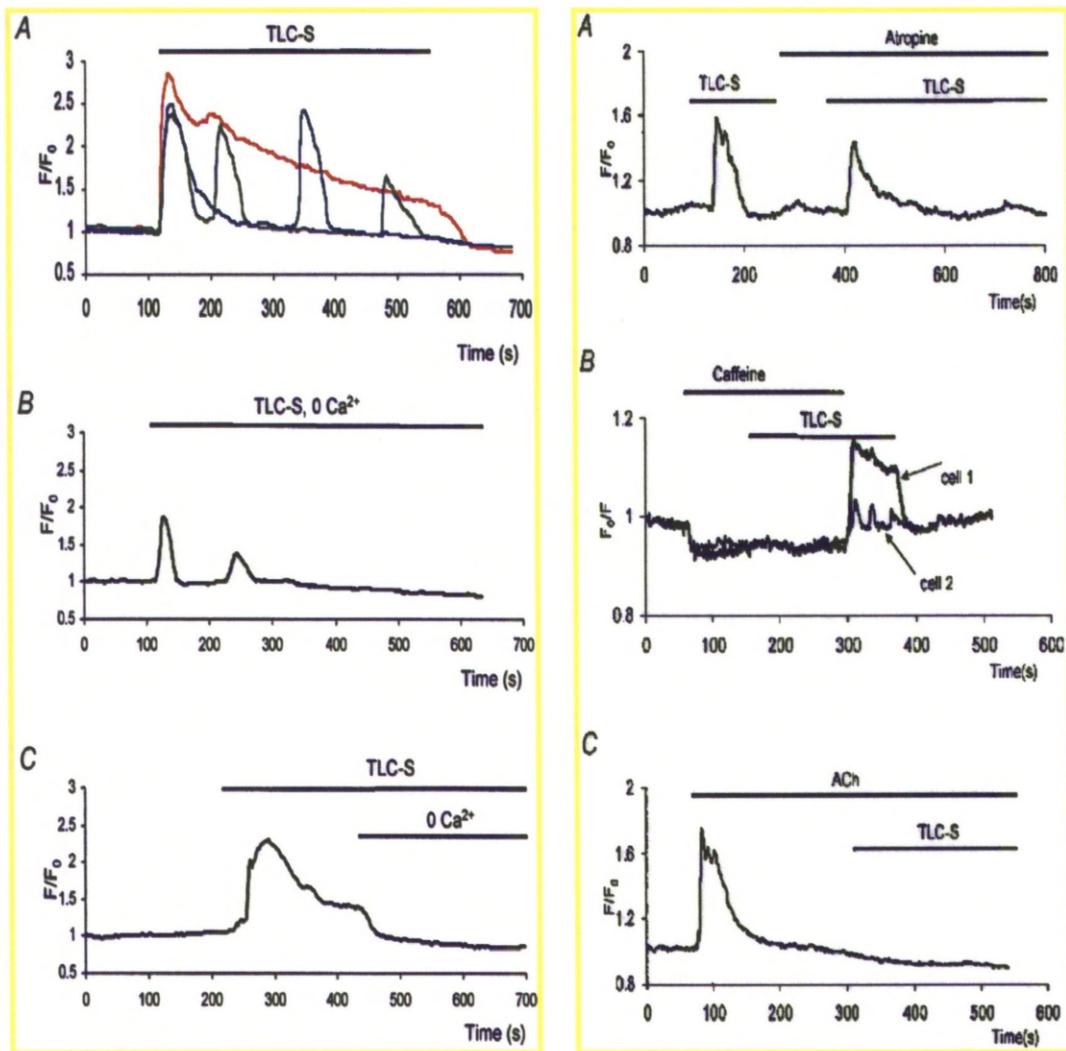


Figure 1.16

Left A, patterns of calcium responses induced by 100 μ M TLC-S in three individual cells (different colours) from the same acinar triplet.

B, calcium responses to TLC-S (200 μ M) in calcium-free external solution.

C, prolonged TLC-S-induced calcium transients were acutely sensitive to removal of calcium from the extracellular solution.

Right: A, atropine (100 μ M) had no effect on TLC-S (200 μ M)-induced release of calcium.

B, caffeine (20 mM) blocked the TLC-S-induced response. Removal of caffeine unmasked the effect of TLC-S. In this experiment, cells were loaded with Fura Red. The fluorescence of Fura Red is only slightly affected by caffeine. Decrease of Fura Red fluorescence corresponds to an increase in the calcium concentration (please note the changes in the scale for this part of the figure).

C, ACh (10 μ M) stimulation is able to completely discharge the TLC-S-sensitive calcium store. The TLC-S concentration was 200 μ M.

From Voronina et al, 2002

Pancreatic acinar cell injury secondary to ethanol and its non-oxidative metabolites

Ethanol consumption is also an extremely important and common cause of acute pancreatitis, since ethanol frequently induces severe disease, may precipitate extensive pancreatic necrosis, and with continued intake, is the commonest cause of progression to chronic pancreatitis (Amman et al, 1996). The earlier postulations of how excessive ethanol consumption induced acute clinical pancreatitis suggested mechanisms whereby proteinaceous deposits plugged into the peripheral ducts causing partial duct obstruction with ductal hypertension and hyper-secretion leading to pancreatitis (Wilson et al, 1989, Pandol et al, 1999).

However, extensive rodent based studies performed recently, examined the cellular events in response to ethanol and subsequent cell fate (Criddle et al 2004, 2006). We now know that ethanol alone and the oxidative metabolite acetaldehyde have minimal or no effects on Ca^{2+} signalling, whereas the non-oxidative metabolites of ethanol - fatty acid ethyl esters induce a profound, concentration-dependent elevation of $[Ca^{2+}]_c$ that results in acinar cell necrosis (Criddle et al, 2004, 2006).

Further recent evidence has also demonstrated rodent acinar cells may be more susceptible to secretagogue induced injury following prolonged exposure to ethanol through a sensitization process (Pandol et al, 1999) which may play an important role in the human form of the disease process.

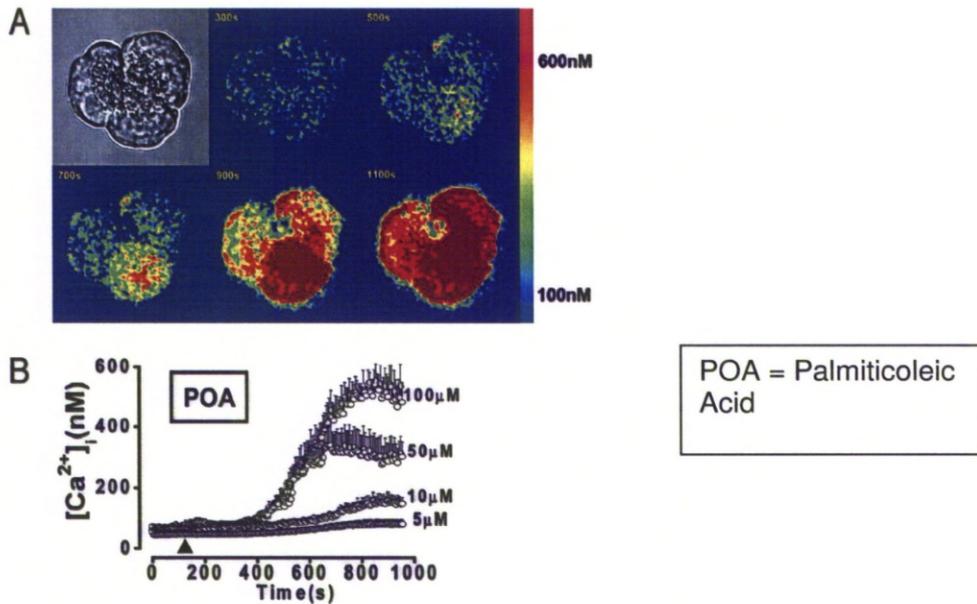
Therefore, current proposed mechanisms in which the pancreatic acinar cell is injured during excessive ethanol ingestion include:

- 1) Pancreatitis induced by non-oxidative metabolites of alcohol (Criddle et al, 2004, 2006) (figures 1.17-1.20)
- 2) Chronic ethanol sensitisation of the acinar cell to naturally occurring stimuli such as CCK or ACh leading to cell death preferentially down a necrosis pathway as opposed to an apoptotic pathway (Letko et al, 1989, Wilson et al, 1989, Pandol et al, 1999),

The pancreas contains high concentrations of non-oxidative synthase enzymes that combine ethanol with fatty acids, forming fatty acid ethyl esters (Laposata et al, 1986, Gukovskaya et al, 2002). There are also high concentrations of hydrolytic esterases that operate in the reverse direction, (Diczfalusy et al, 2001) permitting oxidative metabolism of both products. The increasing evidence that has implicated fatty acid ethyl esters, rather than ethanol itself, to be the cause of pancreatic damage, (Laposata et al, 1986, Gukovskaya et al, 2002, Diczfalussy et al, 2001, Werner et al 1997, 2002, Criddle et al, 2004) has demonstrated Fatty Acid Ethyl Esters (FAEE's), in combination with alcohol, induce Ca^{2+} responses similar to those demonstrated by hyperstimulation (Criddle et al, 2004, Fortunato et al, 2006, Ponappa et al, 1995, Werner et al, 1997, 2001) (figure 1.17). The Ca^{2+} response is produced, in some part, by stimulation of the IP_3 receptor as the Ca^{2+} response is partially blocked by the IP_3 receptor antagonist caffeine (Cancela et al 2000, 2002, Criddle et al, 2004, 2006) (figure 1.18).

Figure 1.17

Palmiticoleic Acid (fatty acid ethyl ester) evokes a global and sustained $[Ca^{2+}]_c$ rise



POA evokes a global and sustained $[Ca^{2+}]_c$ rise. Effects of different POA concentrations on $[Ca^{2+}]_c$. (A) Shown is the typical pattern of $[Ca^{2+}]_c$ changes in an acinar cell triplet (transmitted light image in upper left corner) perfused with 100 μ M POA. An increase in $[Ca^{2+}]_c$ is denoted by a change from a "cold" colour (blue) to a "warmer" colour (through yellow to red) (see colour scale on the right). The Ca^{2+} signal starts in the bottom cell and then spreads, first to the cell on the right and then also to the cell on the left. (B) Dose dependence of mean $[Ca^{2+}]_c$ elevations (vertical bars = SEM) in response to 5, 10, 50, and 100 μ M POA in the presence of external Ca^{2+} .

From Criddle et al, 2004

Figure 1.18

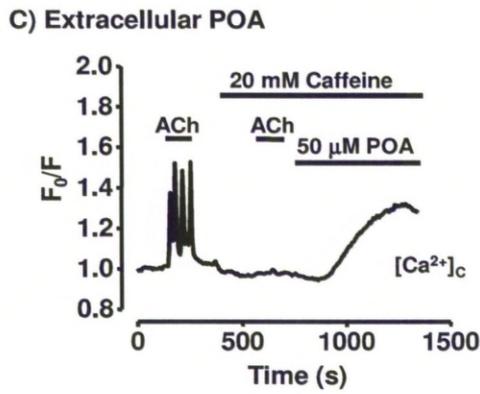
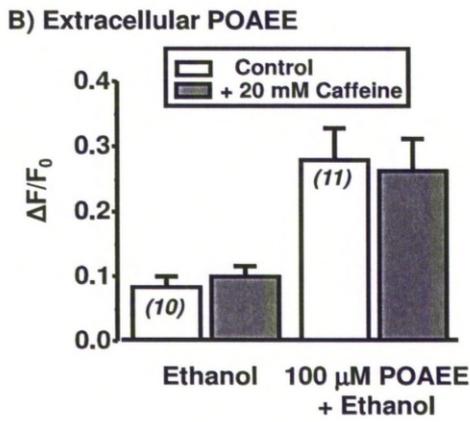
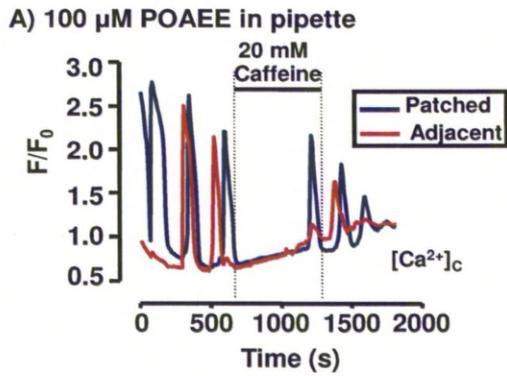


Figure 1.18

A) Typical inhibitory effects of 20 mM caffeine on the oscillatory $[Ca^{2+}]_C$ transients induced by palmitoleic acid ethyl ester (100 μ M POAEE; applied via patch pipette) in a doublet of acinar cells (patched and adjacent cell traces shown). Palmitoleic acid ethyl ester responses were inhibited by caffeine. Ca^{2+} -sensitive Fluo 4 fluorescence is given as the fluorescence ratio (F/F_0),

B) Increases in $[Ca^{2+}]_C$ (measured with Fura Red) induced by ethanol (850 mM) or palmitoleic acid ethyl ester (100 μ M POAEE with 850 mM ethanol), applied extracellularly, are not inhibited by 20 mM caffeine. Data are shown as mean \pm s.e.m., expressed as % changes from the control response before addition of caffeine.

C) Absence of caffeine effect on extracellular palmitoleic acid (POA)-induced $[Ca^{2+}]_C$ elevation, measured with Fura Red. Quasi-physiological (25 nM) acetylcholine (ACh) stimulation evokes characteristic oscillatory increases of $[Ca^{2+}]_C$ that are prevented by 20 mM caffeine through inositol trisphosphate receptor blockade. Continuous caffeine administration does not prevent 50 μ M palmitoleic acid from inducing typical $[Ca^{2+}]_C$ elevation. The Ca^{2+} -sensitive fluorescence of Fura Red is given as the fluorescence ratio F_0/F , since Fura Red fluorescence falls with increasing $[Ca^{2+}]_C$.

From Criddle, Murphy et al, 2006

The FAEE's are hydrolysed to fatty acids within the acinar cell and also cause rapid depletion of ATP resulting in necrosis, a process which is partially reduced by the addition of a hydrolase inhibitor, or by the addition of supplementary ATP (Criddle et al, 2006) (figure 1.19 and 1.20). The FAEE's uncouple oxidative phosphorylation, leading to a global, sustained and toxic increase in $[Ca^{2+}]_c$ due to endoplasmic reticulum calcium leak, through compromise of calcium pump functions in both the endoplasmic reticulum (sarcoplasmic/endoplasmic reticulum calcium ATPase pump, SERCA) and the plasma membrane (plasma membrane calcium ATPase pump, PMCA). The calcium pump activities could be restored by intracellular delivery of ATP restoring the ability of the ATP dependent Ca^{2+} pumps to function again and extrude large concentrations of cytosolic Ca^{2+} into subcellular compartments or externally outside the cell (Criddle et al 2006) (figure 1.19). From the experimental work two important independent actions of the ethanol metabolites were demonstrated:

- (1) Release of calcium from the endoplasmic reticulum is dependent on inositol trisphosphate receptors
- (2) Defective clearance of cytosolic calcium by calcium-ATPase pumps compromised by impaired mitochondrial ATP production.

It is the latter action and its consequent toxicity that were found to depend on the hydrolysis of the fatty acid ethyl ester to liberate its fatty acid by inhibiting mitochondrial function and so ATP production. Indeed using a hydrolase inhibitor to prevent intracellular formation of fatty acid was found to prevent a sustained rise in $[Ca^{2+}]_c$ and allow normal clearance of $[Ca^{2+}]_c$ to continue (figure 1.19).

Figure 1.19

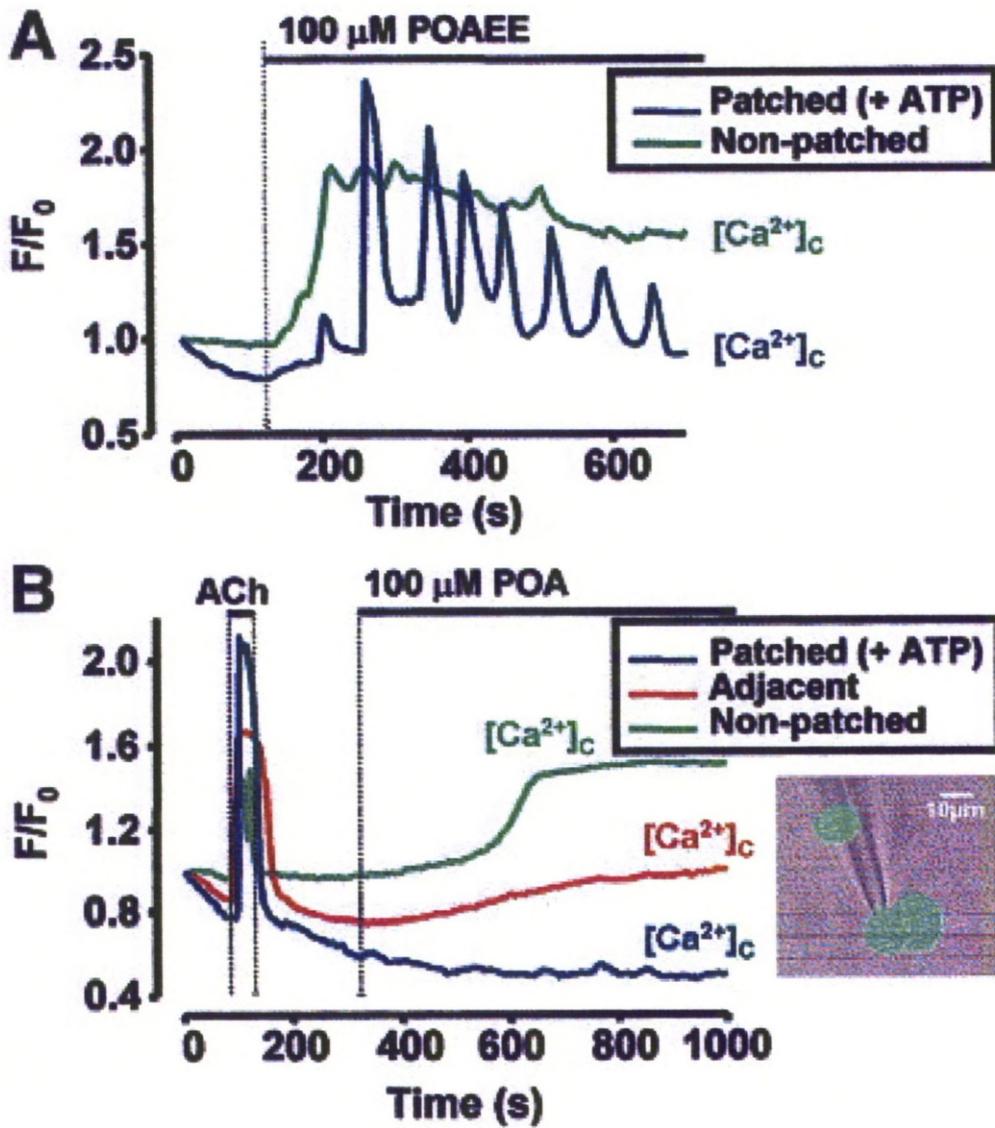


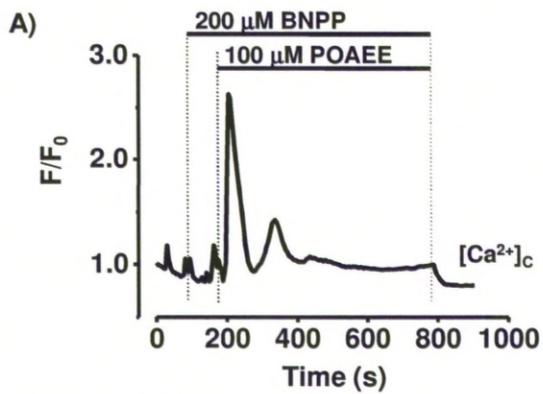
Figure 1.19

A) Representative traces showing the oscillatory elevations of $[Ca^{2+}]_c$ in patched (whole cell recording configuration with direct access from pipette interior to cytosol) cells (ATP in internal pipette solution) induced by extracellular palmitoleic acid ethyl ester (100 μ M POAEE;), compared to the predominantly sustained response observed in non-patched cells.

B) Typical recordings showing the lack of effect of palmitoleic acid (100 μ M POA) on $[Ca^{2+}]_c$ in patched acinar cells with pipette delivery of ATP, compared to non-patched cells which showed a characteristic sustained elevation of $[Ca^{2+}]_c$. $[Ca^{2+}]_c$ is measured by the Fluo 4 fluorescence ratio F/F_0 . Inset shows recorded cells with patch pipette applied to one cell of isolated doublet and separate isolated cell ('non-patched') some distance away.

From Criddle, Murphy et al, 2006

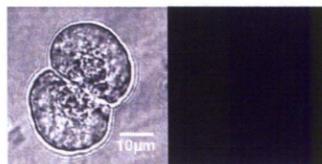
Figure 1.20



B) (i) POAEE



(ii) POAEE + BNPP



(iii)

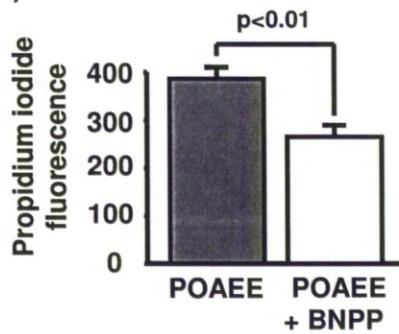


Figure 1.20

A) Typical effect of 200 μM bis-(4-nitrophenyl) phosphate (BNPP), a fatty acid ethyl ester hydrolase inhibitor, to inhibit the sustained elevation of $[\text{Ca}^{2+}]_c$ (measured with Fluo 4) induced by externally-perfused palmitoleic acid ethyl ester (100 μM POAEE;).

B) Typical light-transmitted and propidium iodide fluorescence images of isolated acinar cells after 1 hr incubation with

(i) 100 μM palmitoleic acid ethyl ester alone, showing morphological disruption and propidium iodide staining of the nucleus, and

(ii) 200 μM bis-(4-nitrophenyl) phosphate and 100 μM palmitoleic acid ethyl ester together.

(iii) Propidium iodide fluorescence intensity following staining of cells after 1 hr co-exposure to 100 μM palmitoleic acid ethyl ester and 200 μM bis-(4-nitrophenyl) phosphate, compared to cells exposed to 100 μM palmitoleic acid ethyl ester alone, showing significant protective effects of bis-(4-nitrophenyl) phosphate.

From Criddle, Murphy et al, 2006

The formation of fatty acid ethyl ester requires fatty acid to be present as a cofactor to induce alcoholic pancreatitis. Hyperlipidaemia, a high fat diet and fasting all increase the relative role of fatty acids as metabolic substrates (Rousset et al, 2004, Grundy, 1998) elevating cellular fatty acid concentrations. Fatty acid toxicity, through the mechanisms described, may also be the cause of acute pancreatitis in patients with hyperlipidaemia, which is prevented by effective lipid-regulation (Yaday et al, 2003); also, alcohol is a recognised precipitant of pancreatitis in this group of patients (Durbec et al, 1978, Dickson et al, 2004). Although the sparse epidemiological data on the contribution of a high fat intake to the pathogenesis of alcoholic pancreatitis are conflicting, (Dickson et al, 2004, Wilson et al, 1985) ethanol alone is insufficient for the induction of experimental pancreatitis, while ethanol combined with a high fat diet is a well-documented model (Tsukamoto et al, 1988, Kono et al, 2001). The lack of effect of ethanol contrasts with that of long-chain fatty acid infusion, which results in a severe form of acute pancreatitis characterised by marked ATP depletion, (Nordback et al, 1991) that is central to the pathogenesis of both alcoholic and hyperlipidaemic acute pancreatitis. In addition, alcohol and/or a high fat intake exacerbate both caerulein and bile-induced experimental acute pancreatitis (Katz et al, 1996, Pandol et al, 1999, Lu et al, 2002, Ramo, 1987, Hofbauer et al, 1996). Furthermore, the intermittent excessive consumption of alcohol that is often seen in patients developing alcoholic pancreatitis (Stigendal et al, 1984) may be accompanied by little simultaneous food intake, again leading to an increase in fatty acids as metabolic substrates (Rousset et al, 2004). This suggests that dietary and other appropriate measures that reduce the relative

availability of fatty acids as respiratory substrates could be beneficial in those at risk of pancreatitis from ethanol and its associated metabolites.

This experimental data has identified molecular mechanisms by which ethanol, its non-oxidative metabolites and hyperlipidaemia can induce pancreatic acinar cell injury, and suggest strategies which reduce intracellular fatty acid substrate concentrations may be beneficial in those at risk of pancreatitis.

Also the importance of IP₃ dependent [Ca²⁺]_c release is highlighted and, indeed, coffee consumption may have a modest effect in reducing the risk of alcohol-associated acute pancreatitis most probably related to caffeine – an IP₃R antagonist (Morton et al, 2004). Some other potentially disease relevant findings have been found in that ethanol, can sensitise pancreatic acinar cells to secretagogue (CCK) induced procarboxypeptidase A1 processing in vitro and therefore, can sensitise to various forms of pancreatitis in vivo (Pandol et al, 1999, Ramo et al 1987, Ponappa et al, 1997, Siech et al, 1999). From this we can deduce that although ethanol itself does not induce pancreatitis, it can enhance the stimulation dependent induction of pancreatitis and lead acinar cells to preferentially enter into the necrosis pathway when stressed (Fortunato et al, 2006, Gukovskaya et al, 2004, Satoh et al, 2004, 2006, Wang et al, 2006).

The prolonged ethanol exposure that may sensitise acinar cells to toxic Ca²⁺ release when stimulated by CCK and also to premature NF-κB production as part of the response (Satoh et al, 2006, Wang et al, 2006). It is believed that this is possibly through the Protein Kinase C signalling pathway present in acinar cells, more specifically PKC - ε. PKC is a family of proteins able to release Ca²⁺ from the ER via the production of PLC and DAG in response to G-protein cell surface receptor occupation,

and elegant work published has shown that different isoforms of PKC may be responsible for the sensitization effects of long term ethanol exposure of the acinar cell to receptor stimulation. This may render the acinar cell susceptible to damage, in other wise physiological circumstances.

It has also been hypothesized that long term ethanol exposure has a profound effect on the gene expression. The genes identified as elevated and reduced may contribute to pancreatic sensitivity to stress. The identified genes may explain the relationship between long-term ethanol abuse and pancreatic disease and lead to possible preventative or therapeutic approaches to ethanol-induced acute and chronic pancreatitis (Kubisch et al, 2006).

Is a toxic, sustained $[Ca^{2+}]_c$ rise implicated in rarer precipitants of acute pancreatitis?

One of the rarer, but infamous aetiologies of acute pancreatitis is the scorpion sting. The Trinidadian scorpion *Tityus trinitatis* (Bartholomew, 1970) (figure 1.21) has venom, which acts in a very similar way to an anticholinesterase. It could be postulated, therefore, that hyperstimulation results from the scorpion sting from unopposed vagal stimulation at the acinar cell surface receptor, with an associated sustained intracellular calcium release. Also, the anticholinesterase may cause simultaneous contraction of the sphincter of Oddi with resultant sustained reflux of bile acids down the pancreatic duct with a further stimulus for the sustained rise in the cytosolic Ca^{2+} concentration and premature trypsinogen activation – although at this stage it must be reiterated that the hypotheses have yet to be tested!

Other early animal models of acute pancreatitis induced experimentally used dietary supplements such as arginine and a choline deficient ethionine diet. The pancreatitis witnessed appeared to be due to intraparenchymal activation of zymogens, resulting from a synergistic action of choline deficiency with the basic toxicity of ethionine toward the acinar cells of the pancreas (Lombardi et al, 1975).

Figure 1.21

The scorpion *Tityus trinitatis* indigenous to Trinidad.



Rarely, a scorpion sting can induce acute pancreatitis thought to be due to the action of the anticholinesterases which possibly produces prolonged acetylcholine stimulation of the pancreatic acinar cell and subsequent global sustained $[Ca^{2+}]_c$ release with subsequent premature trypsinogen activation.

Disordered Ca²⁺ signalling, toxic premature trypsinogen activation and vacuole formation

Disordered Ca²⁺ signalling at the apical pole/ secretory granules

The mechanisms whereby disordered Ca²⁺ signalling leads to trypsinogen cleavage remain unclear. Zymogen granules are also Ca²⁺ stores, within which Ca²⁺ maintains granular integrity, and an acidic pH maintains the condensed, inactive state of the enclosed digestive enzymes (Yoo et al, 2000).

Zymogens and other secretory granules release Ca²⁺ through IP₃ and cADP-ribose triggered channels (Gerasimenko OV et al, 1996, Gerasimenko JV et al, 2006, Yoo et al, 2000, Thrower et al, 2005), contributing to the apical cytosolic Ca²⁺ release that leads to exocytosis. Zymogen granule Ca²⁺ release is followed by K⁺ entry (Reeves et al, 2002, Thrower et al, 2005, Petersen OH et al, 2006), accounting for a modest pH increase within maturing granules and preparing enzymes for secretion. If this process is excessive before exocytosis is possible, inappropriate premature enzyme activation may occur (Petersen et al, 2006) due to a marked reduction in pH. With this a confounding element is that as the Ca²⁺ is exchanged for K⁺, the chemically inert precursor enzymes, normal packaged in a crystalline form, may be susceptible to activation as the K⁺ would not be able to support the crystalline structure and unravelling may take place (Nguyen et al,1998) . The ryanodine receptors are thought to be crucial in the process of Ca²⁺ exchange (Husain SZ et al, 2005).

More recent evidence has also implicated endocytosis as potentially important during hyperstimulation. Experimental evidence demonstrated active trypsin being endocytosed back into the cell from the pancreatic ducts during hyperstimulation which contributes to the morphological and pathological features demonstrated on electron microscope during the early phase of acute pancreatitis such as colocalisation of lysosomes with vacuole formation and contribute to the subsequent autophagy with activation of cytokines with the inflammatory cascade of events initiated (Sherwood et al, 2007, Voronina et al, 2007).

Cathepsin B in inappropriate zymogen activation

The role of the lysosomal hydrolase's, more specifically cathepsin B, a cysteine protease, has been hypothesised to be integral to the process of premature intracellular trypsinogen activation, but this is controversial (Halangk et al, 2000).

In the early events of acute pancreatitis, it is postulated that redistribution of intracellular lysosomes in the zymogen rich granular compartment of the cell causes co-localisation and subsequent premature trypsinogen activation in this part of the cell (Hofbauer et al, 2002).

This is supported by work with cathepsin B knockout gene deletion mice and treatments with cathepsin B inhibitors such as E64 showing a reduction in the amount of trypsinogen activation, necrotic cell death and severity of the acute episode when hyperstimulated (Saluja et al, 1997, Gijs et al, 2000). The results suggest that cathepsin B is essential to premature trypsin activation in vitro, but also highlighted:

1) Pancreatitis did still occur in the knockout mice and therefore other mechanisms are involved in premature intracellular activation of trypsinogen,

2) in cathepsin B knockout mice, the induction of pancreatitis had no significant effect on the systemic sequelae such as the systemic inflammatory response syndrome, leucocyte infiltration in the pancreas and lungs or the histological degree of severity (Halangk et al, 2000).

Other investigations into the role of cathepsin B have produced results, which are incompatible with the theory that cathepsin B is vital to premature trypsinogen activation. These include observations such as co localization of cathepsins with digestive zymogens has not only been observed in the initial phase of acute pancreatitis, but also under physiological control conditions and in secretory vesicles that are destined for regulated secretion from healthy pancreatic acinar cells (Tooze et al, 1991).

Other experimental designs using lysosomal inhibitors, found a definite decrease in trypsinogen activation whilst other investigators found both an increase and decrease in premature trypsinogen activation (Leach et al, 1991, Saluja et al, 1997). Indeed one study suggests that cathepsin B activity is not integral, but trypsinogen auto-activation by trypsin itself is the vital process involved (Klonowski – Stumpe et al, 1998).

It is likely that there are several other mechanisms ongoing at the same time and indeed there are also other lysosomal enzymes (cathepsins H and L) (Shikimi et al, 1987) which may play a role in the initiation of premature cleavage of trypsinogen. In comparison to other cell types which may be physiologically relevant, work using cathepsin B and studying liver injury and apoptosis, showed that cathepsin B is thought

to promote cytochrome c release from the mitochondria and induce apoptosis when stimulated with Nuclear Factor kappa B (NFκB). It is of interest that such a mechanism may also be applicable to the pancreatic acinar cell to promote apoptosis rather than necrotic cell death (Guicciardi et al, 2000, 2001).

But at present, there is conflicting evidence of the importance of cathepsin B in the premature activation of trypsinogen which will need further clarification in the future.

Acinar cell secretory block in pancreatic acinar cell injury

Several authors have proposed that it is the malfunction of the exocytic pathway within the acinar cell that renders the cell vulnerable to zymogen granule colocalisation and vacuole formation leading to premature trypsinogen activation. Under circumstances of hyperstimulation with CCK and caerulein, a redistribution of the F - actin cytoskeleton is observed from the sub-apical portion of the cell to the basolateral membrane. With this redistribution, there is a marked inhibition and reduction in exocytotic activity and features of acute pancreatitis are observed, indicating the disruption of the normal exocytotic machinery (Perides et al, 2005, Steer et al, 2004). With the inhibition of exocytosis, colocalisation of subcellular compartments containing lysosomes and zymogens occur leading to premature activation of trypsinogen. It is uncertain if this process is totally Ca²⁺ independent, but studies have shown that the exocytotic process is crucially dependent on the generation of cAMP and Protein Kinase A production, as per secretin stimulation.

Recent evidence, however, has questioned this hypothesis as the F - actin cytoskeleton is redistributed much earlier than cessation of amylase secretion during

hyperstimulation, but in this study the redistribution was unequivocally shown to be independent of disordered Ca^{2+} signalling (Perides et al, 2005).

Pancreatic Ductal cells in acute pancreatitis

Pancreatic ductal cells have been thought to be implicated in acute pancreatitis in patients with cystic fibrosis in whom there is a genetic variation on the cystic fibrosis gene and its product the cystic fibrosis transmembrane conductance regulator on the long arm of chromosome 7 position q31. (Lebenthal et al,1993). This leads to a defective HCO_3^- transported and hence copious thick secretions and cellular debris leading to small ductal obstruction (Lebenthal et al, 1993). Until recently there has been little evidence that the ductal cells have an important role in the pathogenesis of acute pancreatitis from the most common aetiologies.

However, recent work has postulated that while the regulatory pathways that stimulate pancreatic ductal HCO_3^- secretion are well described, little is known about inhibitory pathways, apart from the fact that they exist (Hegyi et al, 2006). These inhibitory pathways may be physiologically important in terms of limiting the hydrostatic pressure within the lumen of the duct, and in terms of switching off pancreatic secretion when not stimulated such as after a meal. Importantly, most of these inhibitors have been shown to reduce secretion in isolated pancreatic ducts, so they are assumed to act directly or indirectly on the ductal epithelium (Hegyi et al, 2006, 2006).

Although this is only a postulation, future work may include testing known precipitants of acute pancreatitis such as bile acids or non-oxidative metabolites of ethanol to ascertain

if they have any direct effects on the ductal cells and possibly inhibit ductal secretion. This could confound the situation by a relative blockade of pancreatic ducts (a reduced amount of secretions would slow down the passage of products of exocytosis into the duodenum), in which activated proteases would be in contact with the pancreatic mass (acinar cells) for a longer period contributing to the mass destruction of the pancreas and future inflammatory response.

Ischaemia, oxidative stress and mitochondrial injury in acute pancreatitis

The majority of the mortality attributed to acute pancreatitis in the early phase (within the first ten days) is secondary to the systemic inflammatory response with associated multi organ dysfunction syndrome.

Oxidative stress with the formation of reactive oxygen species, cytokines and nitric oxide has been identified as being important in assessment of severity in acute pancreatitis. Acute pancreatitis is also caused by mesenteric ischaemia, such as that that may be encountered following coronary artery bypass surgery, or for reasons explained by prolonged periods of hypotension, which also may result in excessive oxidative stress and associated sequelae. Although coronary artery bypass is also associated with intravenous calcium administration which may temporarily cause hypercalcaemia resulting in acute pancreatitis.

Whether ischaemia potentiates acute pancreatitis, or be the sole cause, or the actual ischaemic-reperfusion episode, that follows results in cellular necrosis and destruction is controversial (Isenmann et al, 2004). The reperfusion episode which leads to a massive expression of the numerous cytokines, as well as nitric oxide and other reactive oxygen species triggers not only massive apoptosis, but when the ATP supply in the cell becomes insufficient, necrotic cell death, with the possible subsequent complications (Leindler et al, 2004) such as infection, abscess formation etc. The process then advances further as there is microcirculatory blockage around the pancreas leading to

points of focal hypoxia and subsequent further extensive necrosis (Menger et al, 2001, Uhlmann et al, 2001), by anaerobic respiration.

The specific role of nitric oxide formation in acute pancreatitis and pancreatic necrosis is also unclear in that it may be both protective and destructive, depending on the concentration produced and whether it is produced around or in the acinar cell during the process. Several studies initially suggested that nitric oxide could indeed play a protective role by protecting subcellular fractions of the cell against reactive oxygen species (Sanchez – Bernal et al, 2004), affecting non-acinar cells such as endothelial cells thereby halting progression of the disease (Dimango et al, 2004).

With massive overproduction of nitric oxide, cell necrosis as well as severe acute lung injury results (Leindler et al, 2004, Sandstrom et al, 2004). This has been tested in numerous rodent models using a nitric oxide synthase inhibitor for which several authors have found an improvement in overall survival and histological degree of pancreatic necrosis and acute lung injury (Leindler et al, 2004, Sandstrom et al, 2004, Cheng et al, 2003, Chen et al, 2004).

Oxygen free radicals and reactive oxygen species generated during the acute phase of the disease process damage extracellular tissue by degrading hyaluronic acid and collagen in the intercellular matrix, directly attacking biological membranes through the peroxidation of structurally and functionally important lipids leading to further progression and fuelling of the inflammatory cascade with multi organ dysfunction syndrome. (Byung Kyu Park et al, 2003) (Slater et al, 1984).

In addition, the oxygen free radicals and reactive oxygen species can denature enzymes and other important proteins and damage nucleic acid. They also indirectly

trigger the accumulation of polymorphonuclear (PMN) leucocytes in the tissue, which secrete various enzymes such as myeloperoxidase, protease, and elastase (Babior BM, 1978), resulting in further acceleration of the inflammatory reaction. Oxygen free radicals can also indirectly stimulate arachidonic acid metabolism with increased production of prostaglandins, thromboxane, and leukotrienes, eventually leading to microcirculatory derangement with widespread cellular damage and extensive tissue necrosis.

The evolution of tissue damage in acute pancreatitis involves several inflammatory mediators and an imbalance between pro- and inhibitory inflammatory mediators. The main pro – inflammatory mediators are cytokines.

The cytokine storm in acute pancreatitis

The earliest experimental evidence carried out on dogs by using the Pfeffer preparation (closed loop duodenal obstruction with biliary exclusion) incriminated histamine as a major cause of pancreatitis but it has since become apparent that the histamine release is resultant of the 'cytokine storm' secondary to the initial insult (Schult et al, 1979, Lange et al, 1978).

Acinar cell cytokines (e.g. tumour necrosis factor α , interleukins 1 and 6, platelet activating factor) activation in response to injury is dependent on nuclear factor κ b activation. This is independent of trypsinogen activation and thought to be dependent on abnormal cytosolic Ca^{2+} release.

The cytokines serve to enrol reparative responses and chemokines signal to leukocytes (Han et al, 2000, Bhatia et al, 2002) which in turn enrol a number of proteins and

include the product of acute phase proteins (e.g. C – reactive protein), which is used clinically to indicate the severity of disease. The activation of this cascade directly promotes the systemic inflammatory response in the hyper acute phase.

Several authors have found cytokine expression to occur within 30 minutes of hyperstimulation through prolonged elevation of cytosolic Ca^{2+} (Tnado et al 1999, Han et al, 2000, Bhatia et al, 2002). The exact mechanism of this activation is not well understood as there are several other factors which can stimulate nuclear factor κ b production including isolation of the cell itself (Han et al, 2000, Hieratanta et al, 2001, 2001).

The pro – inflammatory phase of the disease process is then counteracted by the anti – inflammatory mediators such as interleukin 10 and it is this phase of the illness which is responsible for recovery. At this time there is usually a depression in the host immune system and the patient is susceptible to infection with subsequent sepsis and associated mortality (Makhija et al, 2002).

The mitochondria – apoptosis and necrosis in acute pancreatitis

While pancreatic acinar cell injury and death play a major role in pathogenesis, the exact underlying mechanisms have not been fully elucidated. Pancreatic acinar cell death occurs principally via apoptosis or necrosis, the former having protective effects while the latter elicits the inflammatory reaction that can escalate from a local event into a systemic response. Autophagy contributes to intracellular digestive enzyme activation leading to necrosis. The balance between apoptosis and necrosis may act as a fulcrum which determines the severity of acute pancreatitis.

Normal mitochondrial function

Mitochondrial metabolism is essential for the production of usable energy in the normal cell. The word mitochondrion comes from the Greek *mitos* - thread and *chondrion* - granule. The mitochondrion is found in most eukaryotic cells, range from 0.5 to 10 micrometers (μm) in diameter and generate most of the cell's supply of adenosine triphosphate (ATP) (Henze et al, 2003). In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signalling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth (McBride et al, 2006). Several characteristics make the mitochondrion unique in that they are composed of compartments that carry out specialized functions. These compartments include the outer membrane, the intermembrane space, the inner membrane, and the cristae and matrix. The outer and inner membranes are composed of phospholipid bilayers and proteins (Alberts et al, 1994) but have different properties.

The outer mitochondrial membrane contains large numbers of integral proteins called porins. These porins form channels that allow molecules 5000 Daltons or less in molecular weight to freely diffuse from one side of the membrane to the other (Alberts et al, 1994). Larger proteins can enter the mitochondrion if a signalling sequence at their N-terminus binds to a large multi subunit protein called translocase of the outer membrane, which then actively moves them across the membrane (Hermann et al, 2000). Disruption of the outer membrane permits proteins in the intermembrane space to leak into the cytosol, leading to certain cell death. In the intermembrane space, the concentrations of small molecules, such as ions and sugars, are the same as the cytosol (Alberts et al, 1994). However, large proteins must have a specific signalling sequence to be transported across the outer membrane, so the protein composition of this space is different from the protein composition of the cytosol. One protein that is localized to the intermembrane space in this way is cytochrome c. The inner mitochondrial membrane contains proteins with five types of functions (Alberts et al, 1994):

1. Those that perform the redox reactions of oxidative phosphorylation
2. ATP synthase, which generates ATP in the matrix
3. Specific transport proteins that regulate metabolite passage into and out of the matrix
4. Protein import machinery.
5. Mitochondria fusion and fission protein

The matrix is the space enclosed by the inner membrane. It contains about 2/3 of the total protein in a mitochondrion (Alberts et al, 1994). The matrix is important in the production of ATP with the aid of the ATP synthase contained in the inner membrane. The matrix contains a highly-concentrated mixture of hundreds of enzymes, special mitochondrial ribosomes, tRNA, and several copies of the mitochondrial DNA genome. Of the enzymes, the major functions include oxidation of pyruvate and fatty acids, and the citric acid cycle/ Krebs's cycle with production of ATP through aerobic respiration (Alberts et al, 1994). More precisely this is done by oxidizing the major products of glucose, pyruvate, and NADH, which are produced in the cytosol (Voet et al, 2006) and is dependent on the presence of oxygen. When oxygen is limited, the glycolytic products will be metabolized by anaerobic respiration, a process that is independent of the mitochondria (Voet et al, 2006). The production of ATP from glucose has an approximately 13-fold higher yield during aerobic respiration compared to anaerobic respiration.

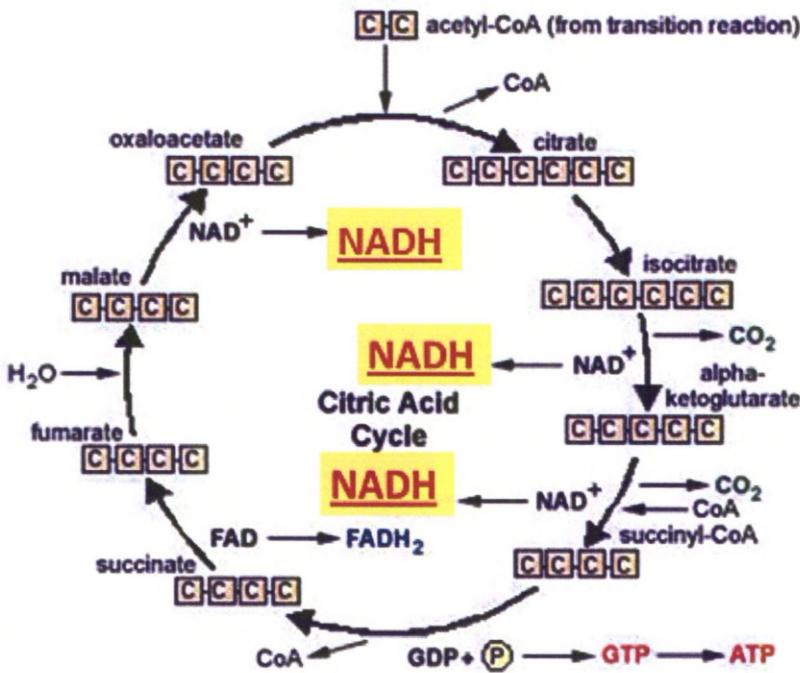
Pyruvate and the citric acid cycle

Each pyruvate molecule produced by glycolysis is actively transported across the inner mitochondrial membrane, and into the matrix where it is oxidized and combined with coenzyme A to form CO₂, acetyl-CoA, and NADH. The acetyl-CoA is the primary substrate to enter the citric acid cycle. The enzymes of the citric acid cycle are located in the mitochondrial matrix, with the exception of succinate dehydrogenase, which is bound to the inner mitochondrial membrane as part of Complex II (King et al, 2006). The citric acid cycle oxidizes the acetyl-CoA to carbon dioxide, and, in the process,

produces reduced cofactors (three molecules of NADH and one molecule of FADH₂) that are a source of electrons for the electron transport chain, and a molecule of GTP (that is readily converted to an ATP) (figure 1.22) . The redox energy from NADH and FADH₂ is transferred to oxygen (O₂) in several steps via the electron transport chain. Protein complexes in the inner membrane (NADH dehydrogenase, cytochrome c reductase, and cytochrome c oxidase) perform the transfer and the incremental release of energy is used to pump protons (H⁺) into the intermembrane space. This process is efficient, but a small percentage of electrons may prematurely reduce oxygen, forming reactive oxygen species such as superoxide (Voet et al, 2006). This can cause oxidative stress in the mitochondria and may contribute to the decline in mitochondrial function. As the proton concentration increases in the intermembrane space, a strong electrochemical gradient is established across the inner membrane. The protons can return to the matrix through the ATP synthase complex, and their potential energy is used to synthesize ATP from ADP and inorganic phosphate (Pi). Mitochondria play a central role in many other metabolic tasks including:

- Apoptosis-programmed cell death (Baumgartner et al, 2007)
- Calcium signalling (including calcium-evoked apoptosis) (Baumgartner et al, 2007, 2009)

Figure 1.22



Schematic of the krebs/citric acid cycle: The total number of ATP obtained after complete oxidation of one glucose in glycolysis, citric acid cycle, and oxidative phosphorylation is estimated to be between 30 and 38 ATP molecules. NADH is produced at several steps during the cycle which can be observed/monitored using UV laser excitation and emission whilst observing fluorescent dye changes concurrently with confocal microscopy as detailed in the next chapters.

Apoptosis and necrosis in pancreatic acinar cells

When considering apoptotic (programmed) cell death in the pancreatic acinar cell it is first worth describing the function of the mitochondrial permeability transition and the mitochondrial transmembrane protein transition pore. Mitochondrial permeability transition, or MPT, is an increase in the permeability of the mitochondrial membranes to molecules <1.5 kDa. MPT results from opening of mitochondrial permeability transition pores (MPTP). The MPTP opens under certain pathological conditions inducing cell stress such as a toxic $[Ca^{2+}]$ elevation (Gerasimenko et al, 2009). Induction of the permeability transition pore can lead to mitochondrial swelling with cell death and plays an important role in apoptosis. MPT may also play a role in mitochondrial autophagy (Leamasters et al, 1998). Cells exposed to toxic amounts of $[Ca^{2+}]$ can also undergo MPT and opening of the MPTP leading to death by necrosis rather than apoptosis. This is possibly because Ca^{2+} binds to and activates Ca^{2+} binding sites on the matrix side of the MPTP (Haworth et al, 1979, Ichas et al, 1998). MPT induction is also due to the dissipation of the difference in voltage between the inside and outside of mitochondrial membranes – mitochondrial membrane potential ($\Delta\psi_m$) (Armstrong et al, 2004, Schinder et al, 1996). The presence of free radicals, another result of excessive intracellular calcium concentrations, can also cause the MPT pore to open (Baumgartner et al, 2009). Mechanisms that cause the pore to close or remain closed include acidic conditions (Friberg et al, 2002), high concentrations of ADP (Haworth et al, 1979), high concentrations of ATP (Beutner et al, 1998), and high concentrations of

NADH (Criddle et al, 2007). The induction of MPT, which increases mitochondrial membrane permeability, causes mitochondria to become further depolarized, meaning that $\Delta\psi_m$ is abolished. When $\Delta\psi_m$ is lost, protons and some molecules are able to flow across the outer mitochondrial membrane uninhibited (Schinder et al, 1996). Loss of $\Delta\psi_m$ interferes with the production of adenosine triphosphate (ATP), the cell's main source of energy, as the mitochondria must have an electrochemical gradient to provide the driving force for ATP production. MPT also allows Ca^{2+} to leave the mitochondrion, which can place further stress on nearby mitochondria with subsequent ATP depletion. In addition, the electron transport chain (ETC) may produce more free radicals due to loss of components of the electron transport chain (ETC), such as cytochrome c, through the MPTP. As the MPT causes mitochondria to become permeable to molecules smaller than 1.5 kDa, this can result in increasing the organelle's osmolar load as the smaller molecules pass to the inside of the organelle (Buki et al, 2000, Criddle et al, 2007). This leads mitochondria to swell with possible outer membrane rupture, releasing cytochrome c (Buki et al, 2000). Cytochrome c can in turn cause the cell to go through apoptosis by activating pro-apoptotic factors. Much research has found that the fate of the cell after an insult depends on the extent of MPT. If MPT occurs to only a slight extent, the cell may recover, whereas if it occurs more it may undergo apoptosis. If it occurs to an even larger degree the cell is likely to undergo necrotic cell death.

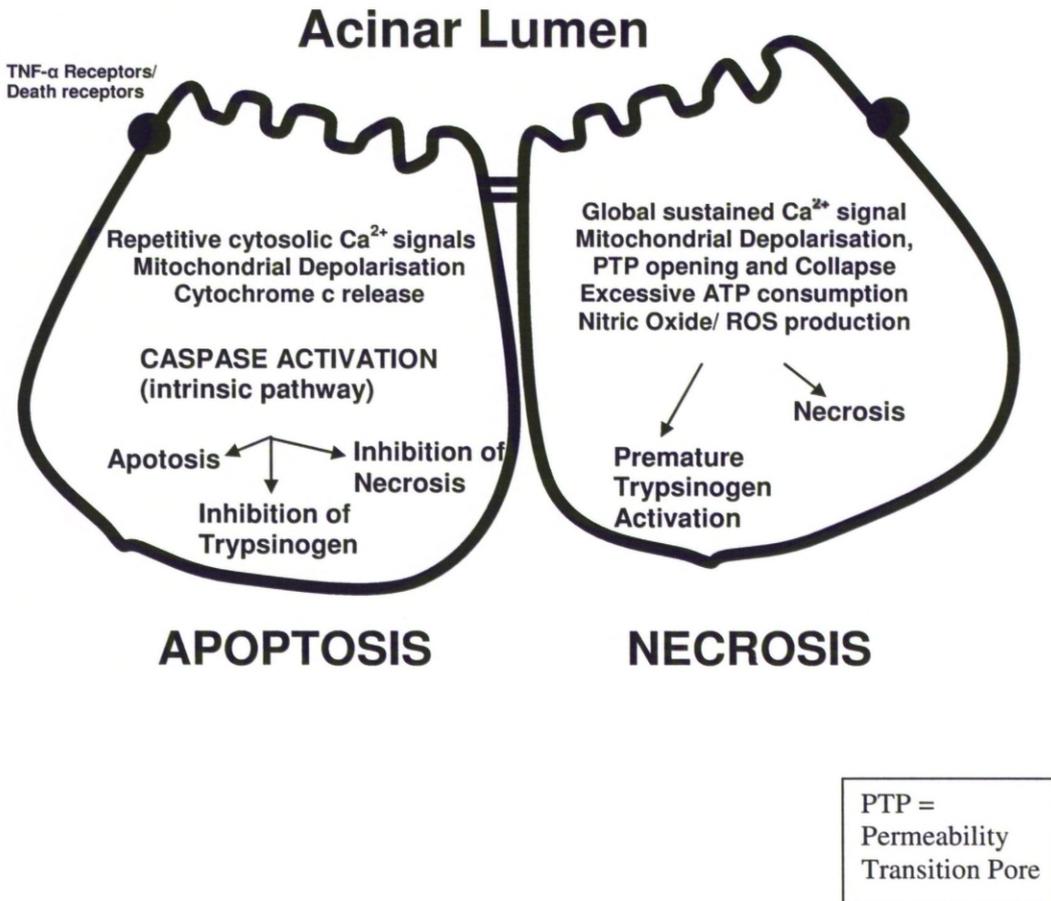
Previous studies demonstrated menindione; an oxidant activates the MPTP causing partial mitochondrial depolarisation (Gerasimenko et al, 2002). This results in a loss of ability of the peri-nuclear mitochondria to delay Ca^{2+} entry into the nucleoplasm,

cytosolic Ca^{2+} release and results in activation of caspases and cytochrome c within the cytosol and the acinar cell proceeds along the apoptotic pathway (Gerasimenko et al, 2002, Baumgartner et al, 2008). Recently however, elegant studies have demonstrated that it is indeed the elevation of Ca^{2+} which is the major requirement in the pancreatic acinar cell for MPTP opening and apoptotic cell death through the cytochrome c/ caspase dependent intrinsic pathway (Baumgartner et al, 2009).

The process determining whether the acinar cell proceeds along the apoptotic or the necrotic cell death pathway is unclear and poorly understood. It has been suggested that promotion of necrosis through ATP depletion might in part be mediated via an inability of the apoptosome to activate the initiator caspase 9 (Baumgartner et al, 2008). Other explanations include that if there were greater interference, such as that observed with a global sustained intracellular Ca^{2+} rise from a toxic stimuli, cell death would be accelerated without adequate ATP generation, and necrosis rather than apoptosis would result (Petersen et al, 2006) activating a number of mechanisms including cell surface receptors for NF κ B, TNF α and p53 (Bhatia et al, 2004) (Figure 1.23). Therefore, cell injury resulting from inappropriate activation of enzymes and subsequent digestion, coupled with mitochondrial membrane depolarization and stimulation by TNF- α , NF κ B and p53, results in cell necrosis of which the extent and severity culminates in clinically apparent acute pancreatitis, but the correlation between the extent of the pancreatic cell death and clinical severity is not well understood (Mukherjee et al, 2008).

Figure 1.23

Schematic diagram of pancreatic acinar cell apoptotic (via caspase activation through the intrinsic pathway) and necrotic cell death pathways.



Cell stress and reactive oxygen species generation

Reactive oxygen species (ROS) have also been implicated in the pathogenesis of pancreatitis. Repeated experiments demonstrated pathological peroxidation products and depletion of endogenous antioxidants early in the course of disease (Weber et al, 1998, Tsai et al, 1998); in these models many anti-oxidant treatments, significantly reduce inflammation and pancreatic tissue injury. Elevated levels of ROS and antioxidant depletion are found within pancreatic tissue and the systemic circulation in clinical pancreatitis. However, clinical trials using antioxidant treatment have produced conflicting results and have even been stopped because excess adverse events occurred in association with the treatment (Johnson et al, 2001). Therefore, as yet it is unclear whether ROS generation is harmful to the pancreatic acinar cell during pathological stimuli.

A final point of interest in the pathogenesis is Ca^{2+} depletion of the endoplasmic reticulum is a recognised source of cell stress. The result of which is mis-folding of proteins and impaired export through the Golgi apparatus. Excessive accumulation of the mis-folded proteins in the endoplasmic reticulum has been hypothesised to cause further Ca^{2+} release into the cytosol and induce further expression of nuclear factor kb via reactive oxygen species (Paschen, 2001, Pahl HL, 1999). Further still, neutrophils and neutrophil elastase have been identified as a marker of systemic disease severity as it is thought that neutrophil infiltration in the pancreas itself, the liver, kidneys and lungs contributes to the systemic inflammatory response. Neutrophil elastase inhibitors demonstrated in vivo in rodents a marked reduction in disease severity both at a

localised level within the pancreas and a reduction in the severity of acute lung injury (Paschen, 2001, Pahl HL, 1999).

Current and future directions in acute pancreatitis

Although considerable progress has been made into the cellular basis of acute pancreatitis, the picture is by no means complete. Several randomised controlled trials based on treatment of complications have failed to improve the morbidity and mortality, including studies with the early use of antibiotics (Isenmann et al, 2004, Raraty et al, 2004), the use of drugs reducing pancreatic exocrine function (Niederau et al, 1993), as well as anti inflammatory drugs and drugs targeting specific cytokines. Several models have been shown to be beneficial in vitro but unfortunately this has not transferred in human clinical acute pancreatitis and we await the results of newer therapies being tested at present or in the near future (Makela et al, 1993, Song et al, 2002, Yang et al, 2004, Yamigiwa et al, 2004).

However, it has been shown that the use of non-steroidal anti inflammatory drugs is beneficial to patients undergoing endoscopic retrograde cholangiopancreatography in prevention of acute pancreatitis resulting from ERCP (Murray et al, 2003).

Although there have been several studies looking at the systemic inflammatory response to the disease process, there is no effective therapeutic target established which reduces the mortality in the hyper acute phase of the disease process. Therefore targeted future work must be performed to observe whether sequelae of acute pancreatitis such as the cytokine storm can be manipulated in a way to reduce effectively the multi organ dysfunction syndrome that is associated with those patients

few who develop the disease in its severe form. From the array of trials that has passed before us, it is apparent that the therapeutic intervention is not early enough in the evolution and progression of the disease. An important step is to assess if and where the chain of events can be manipulated, both in vitro and in vivo at the very earliest stage.

Ultimately, an ideal therapeutic target would be able to be administered upon admission to the emergency room, and this is why understanding is needed in the earliest pathological events within the acinar cell to treat before the ensuing cytokine storm and subsequent detrimental effects caused.

We must also begin to undertake research on human pancreatic acinar cells as the majority of work undertaken so far has been on animal pancreatic acinar cells for obvious technical and ethical reasons (Miller LJ, 1996). Indeed, there have been exceedingly few experimental models of acute pancreatitis published using human pancreatic acinar cells. However, interestingly, the experiments demonstrated have cast some doubt as to the role of hyperstimulation with CCK as a model of acute pancreatitis, as it is postulated that human pancreatic acinar cells do not directly respond to Cholecystokinin, but via a vagal loops (Ji et al, 2001, 2002, Owang et al, 2004), a point that we have demonstrated to be inaccurate in the chapters below (Murphy et al, 2008). Therefore more studies are needed using animal pancreatic acinar cells to understand the very earliest pathological events and then translate this work into human pancreatic acinar cells to examine if the same processes are replicated. Only then can effective therapeutic agents be administered to ameliorate the potentially disastrous consequences of severe acute pancreatitis.

Hypotheses and study aims:

1) Cholecystokinin (CCK) has been implicated to act only indirectly on human pancreatic acinar cells via vagal nerve stimulation.

The null hypothesis is that CCK cannot act, via specific cell surface receptors (CCK A), on human pancreatic acinar cells to produce both physiological and pathological intracellular Ca^{2+} signalling.

The aims were to test whether CCK (CCK-8 and human CCK-58) can act directly on human pancreatic acinar cells.

2) Sustained, toxic elevations of the acinar cytosolic free calcium ion concentration ($[Ca^{2+}]_c$) are implicated in acute pancreatitis as demonstrated by supramaximal stimulation. However, clinically, such stimulation is unlikely to occur due to the large concentrations of stimulus needed to produce the toxic effect. Many patients have eaten prior to the onset of pancreatitis and it may be a combination of neurohumoral and lower concentrations of toxic stimuli that precipitate the earliest pathological intracellular events.

The null hypothesis is that normal concentrations of secretagogue together with low dose toxin stimulation do not initiate toxic, sustained intracellular calcium signals and energy dependent cell death.

The aims were to test whether lower doses of secretagogues and toxins can produce toxic intracellular calcium signals and ascertain how the toxic signals are initiated which lead to cell death.

Chapter 2

Materials and Methods

Solutions

All chemicals, unless specified, were purchased from Sigma Aldrich (Gillingham, UK) and were of the highest grade available.

Standard extracellular solution

The extracellular solution used throughout acinar cell preparation and during all experiments contained the following chemicals dissolved in distilled H₂O:

	<u>Concentration (mM)</u>
NaCl	140
KCl	4.7
MgCl ₂	1.13
D-glucose	10
Hepes	10
CaCl ₂	1

The pH was adjusted to 7.40 with NaOH (5M) and the osmolality checked with an osmometer to be 300 ± 10 mOsm. CaCl₂ and other intracellular agents were adjusted as and when required and are noted in the descriptions of individual experiments. The agents used during experimentation were diluted in this solution from stock solutions, which were prepared along manufacturers guidelines.

Standard internal (intra-pipette) solution

Standard whole-cell patch-clamp recordings (as described below) were performed using an internal (intrapipette) solution, to mimick the intracellular cytosolic environment. This solution contained the following chemicals dissolved in distilled H₂O:

	<u>Concentration (mM)</u>
KCl	140
NaCl	10
MgCl ₂	1.5
Hepes	10
EGTA	0.1

The pH of the solution was 7.2 (adjusted with 5M KOH), with an osmolality of 300 ±10 mOsm. Where stated, a standard concentration of 2mM MgATP was present, or an elevated concentration of 4mM MgATP, or MgATP was omitted (0mM) from the internal solution. The solution was then filtered through a 0.2µl Millipore filter syringe and then stored at -20°C. Where stated, Inositol Trisphosphate (IP₃) at a physiological concentration of 1-10µM, Cyclic Adenine Diphosphate Ribose (cADPr) 10-15µM or Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP) 50-100nM was added to the internal solution (all chemicals from Calbiochem, Germany).

Collection of Human Pancreatic Tissue Samples

All human pancreatic tissue samples used during experiments were obtained from patients undergoing pancreatic resection at the Royal Liverpool University Hospital, Liverpool, UK, between January 2004 and February 2008.

Ethical approval and patient consent

The study was approved by the Liverpool Adult Local Research Ethics Committee (Ref: 03/12/242/A). On the day before the patient was due to undergo the appropriate pancreatic resection, written informed consent for sampling of macroscopically normal pancreas from the transection margin during the operative procedure was obtained.

Human Pancreatic Tissue Sample Retrieval

During surgery (pancreatic resection), a small piece (~1 cm² x ~1 mm) of pancreas was cut from the transection margin of the remaining pancreas, initially with diathermy, but in later cases with a new scalpel blade to limit gross macroscopic cell damage. The sample was immediately washed of debris and blood products and taken to the laboratory in the extracellular solution described above, on ice. Latterly complete protease inhibitors (ROCHE, USA) and the cell membrane permeant trypsin inhibitor, benzamidine (1mM) (Worthington Biochemical, Lakewood, NJ), were added to this solution, again to limit cell damage. The time from sampling to the start of cell isolation was less than 10 minutes in every case.

Preparation of pancreatic acinar cells

Murine pancreatic acinar cell isolation

Freshly isolated murine pancreatic acinar cells, acinar cell doublets or acinar cell clusters were obtained from pancreases of adult male CD1 mice (weight 30 – 35g and aged between 21-30 days) by collagenase digestion (Worthington Biochemical Corporation, Lakewood, NJ). Before removal of the pancreas, mice were stunned by a blow to the head and killed by cervical dislocation in accordance with the Animal (Scientific Procedures) Act, 1986. A laparotomy was then performed to expose the abdominal organs from which the pancreas could be identified easily as being attached to the spleen. Careful sharp dissection was performed to remove the pancreas from its ligamentous attachments to the spleen and placed immediately into the extracellular solution. The pancreatic tissue was then injected, via the main pancreatic duct, with collagenase 200U/mL (Worthington, Lorne Laboratories, Reading, UK) and incubated in a water shaking bath at 37^oc for 13 -15 minutes. Following digestion, the pancreas was placed into a Falcon tube containing around 4 - 5ml standard extracellular solution. Manual agitation and dispersal through pipette tips of progressively diminishing diameter produced the final population of pancreatic acinar cells, which were resuspended twice in an extracellular solution buffer following a period of 1 minute centrifugation at 1000 revs per minute in each instance. The final cell pellet was resuspended in 2ml extracellular solution. All experiments were performed at room temperature and cells used within three hours of isolation.

Human pancreatic acinar cell isolation

Single acinar cells and acinar cell clusters were isolated by a method adapted and modified from the rodent work outlined above. Briefly the section of pancreatic tissue was manually dissected using fine forceps until all non-pancreatic tissue was removed from the sample. The sample was then injected at several points with collagenase (200 U/ml) (Worthington, Lorne Laboratories, Reading, UK). Unlike the mouse pancreas, delivery to every portion of the pancreatic sample was impossible as there usually was not an intact pancreatic ductal system following the initial surgical resection of the sample. To attempt to overcome this, the sample was macerated into fine pieces using a fresh surgical blade (size 15) so that the surface area exposed to the collagenase was as large as possible. The sample was then incubated in an agitating water bath for 15 – 30mins at 37°C. The procedure was then similar to that used for isolated mouse pancreatic acinar cells as described above.

In the early stages of human pancreatic acinar cell preparation, the cells isolated bared little resemblance to what was regularly obtained when isolating mouse acinar cells. As the cells looked damaged morphologically (and subsequently proved by positive BZiPAR fluorescence indicating massive premature trypsin activity), the isolation process was adapted with the following modifications:

- i) Resection techniques; the Pancreatic Surgeon undertaking the surgical resection obtained the sample by dissection using a fresh blade – avoiding the use of destructive diathermy burns in such small samples, a technique which did not cause detrimental blood loss to the patient

- ii)** Transport solution; the sample was washed three times to remove surface blood from the tissue which may contain neutrophils, macrophages etc. which have the potential, in theory, to induce additional oxidative stress in the sample before it could be cooled for transportation. Pyruvate was added to the transport solution to ensure the mitochondria could function as well as possible in the absence of a good blood supply. The sample was also transported in an extracellular solution containing complete protease inhibitors (ROCHE, USA) and 1mM Benzamidine, (Worthington Biochemical, Lakewood, NJ) a cell membrane permeant trypsin inhibitor to try to avoid any premature intracellular trypsinogen activation and features of acute pancreatitis.
- iii)** Isolation technique; the pancreatic sample was injected at several points with collagenase (200 U/ml) with 1mM benzamidine and the tissue was finely minced and incubated at 37 °C for 15– 30mins, collagenase drained, and tissue suspended in the extracellular solution as previously described. The suspension was triturated and supernatant collected; this was repeated until the supernatant was clear. Cells in the supernatant were pelleted by centrifugation at 1000 revs/min for 1 min, triturated again in fresh extracellular solution, filtered (70 µm Cell Strainer, BD Biosciences, Bedford, MA) to remove undispersed tissue, and centrifuged as before. The final pellet was re-suspended in the extracellular solution and all experiments were performed at room temperature within 2 hours.

Cell imaging

Confocal Microscopy

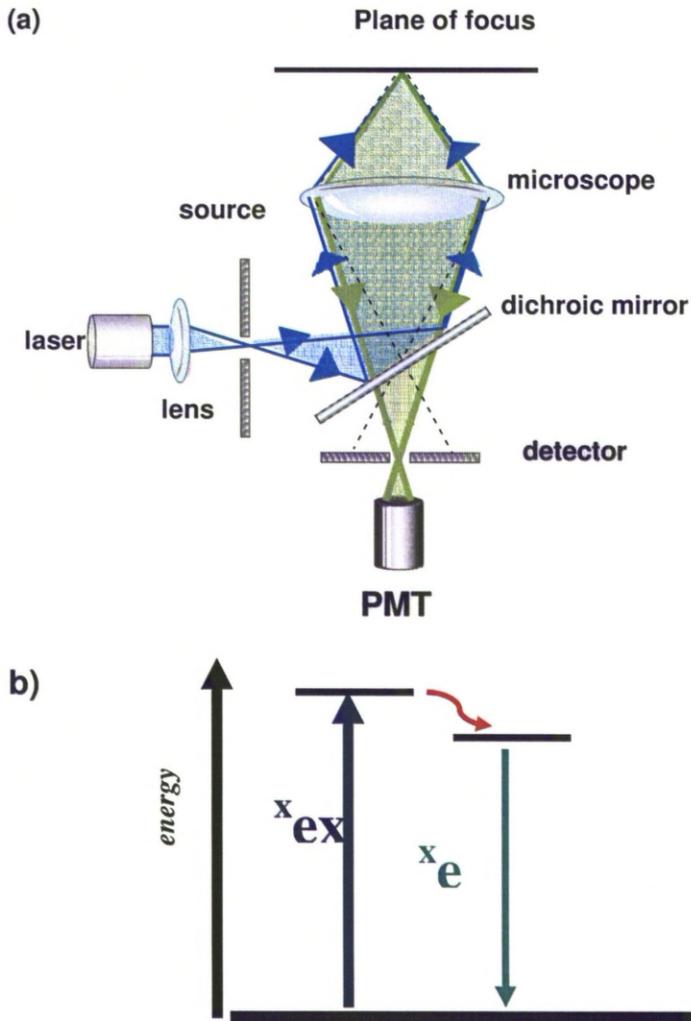
The resolution of fluorescence microscopy is largely limited by interference from so-called "out of focus light". Normally, in fluorescence microscopy, when an object is imaged, the signal produced results from the entire thickness of the sample and thus does not allow the majority of it to be in focus. This results in loss of spatial information, to an extent that small and discrete fluorescent signals, for example, small localised Ca^{2+} signals produced in pancreatic acinar cells, become difficult to assess accurately. The spatial resolution may be improved by using confocal microscopy. This method offers several distinct advantages over conventional imaging techniques including the ability to control depth of field, elimination of out of focus light thereby restricting the fluorescent measurements to the focal plane and the capability to collect serial optical sections from the thick specimens.

The principle of confocal microscopy is presented in figure 2.1. The excitation light, provided by a laser light source, passes through the excitation pinhole and is reflected by the dichroic mirror towards the microscope objective. The objective focuses the excitation light onto a point within the sample, where it excites fluorophores within the fluorescent indicator/protein, raising them to an excited state. The process of absorption is rapid and is followed immediately by a return to a lower energy state that is accompanied by emission of light as part of this process. The light emitted from the focal point is collected by the objective and passes through the dichroic mirror. This is possible as the emitted light is of a lower energy and therefore of a longer wavelength

(Stoke's shift) and therefore allows excitation and emitted light to be easily distinguished. Having passed through the dichroic mirror, the emitted light is focused onto a pinhole positioned in front of the photomultiplier tube (PMT). This emission pinhole acts as a spatial filter, allowing only the in-focus portion of the light to pass through, whilst providing a barrier to light above and below the focal plane. It is at this point that the focus of light corresponds exactly to the point of focus on the sample and is therefore "confocal". The focused light is able to pass through the pinhole where it is detected by the PMT. By altering the angle of the scanning mirrors, the focal point can be moved in the 'xy' plane allowing the whole specimen to be scanned. Additionally, the emission pinhole can be widened to increase the depth of the optical slice. By altering the plane of focus, a series of confocal sections can be collected and with the use of computer software, can be reconstructed to produce a three dimensional image of the sample.

The confocal systems used for fluorescence imaging were a Zeiss LSM 510 system and a Leica TCS SP2 fitted with an acoustical optical beam splitter (AOBS). Both systems are laser-scanning microscopes fitted with ultra violet (Enterprise II, Coherent) and visible lasers (HeNe, Argon) to provide excitation sources. Fluorescent signals were recorded on the stage on inverted using a 63x water immersion objective lens with a numerical aperture of 1.2. Excitation laser lines and emission wavelengths were selected according to manufacturer's guidelines (see table 2.1)

Figure 2.1



a) Schematic illustrating the pathway of excitation (blue) and emission (green) light in confocal microscopy.

b) Jablonski energy diagram demonstrating single photon excitation (confocal) excites the fluorophores, raising it to a higher energy level. It then rapidly falls back to a lower energy state emitting light of a longer wavelength.

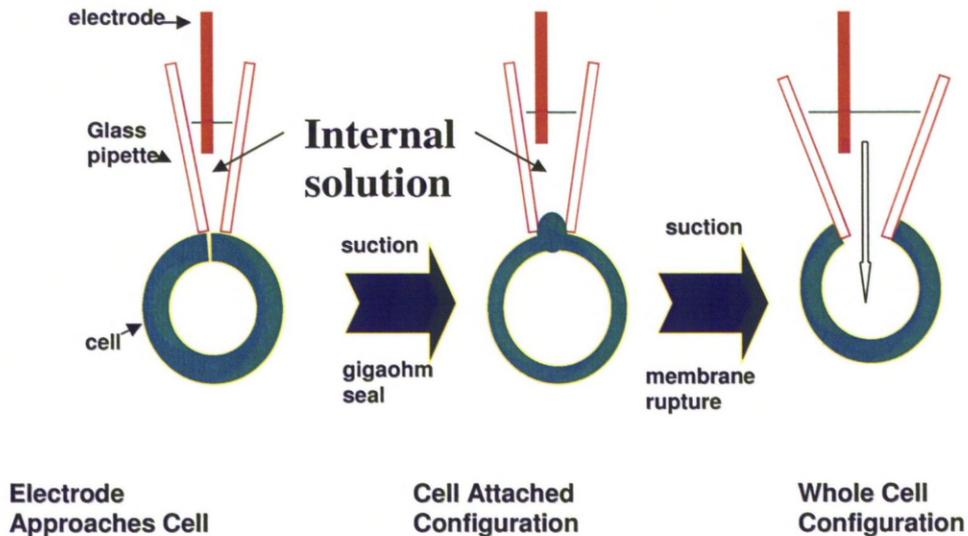
Electrophysiology

Patch clamp recordings

Cells were investigated using the whole-cell patch-clamp configuration (Hamill et al, 1981) as demonstrated in figure 2.2 From a holding potential of -30mV, steps were made to 0mV, the reversal potential of the two Ca²⁺-dependent currents through Cl⁻ and non-selective cationic channels (Criddle et al, 2006). Using our solutions, the reversal potential of both the Cl⁻ and non-selective cation currents was at 0mV (for use as a potential control, Cancela et al, 2000). Small deviations in E_{Cl} and E_{cation} and in the holding potential sometimes produce small inward or outward currents at 0mV. At -30mV a measure of both Ca²⁺-dependent currents were obtained, which are an index of the cytosolic Ca²⁺ changes (Cancela et al, 2000, Criddle et al, 2006). Patch pipettes were pulled from borosillate glass capillaries (Harvard Apparatus, Edenbridge, Kent,UK) with a resistance of 2.5–3.5 MΩ when filled with the intracellular solution. Whole cell currents were sampled at 10KHz using an EPS8 amplifier and Pulse software (HEKA, Lambrecht, Pfalz, Germany) as demonstrated in figure 2.4

Figure 2.2

Principles of the whole cell patch clamp technique



Schematic showing the patch clamp technique used to generate a 'giga ohm' seal in whole cell configuration so that experiments investigating the second messengers and differing intracellular ATP concentrations could be undertaken:

- the patch pipette containing standard internal solution with or without second messenger and ATP approaches the pancreatic acinar cell under direct vision;
- Gentle suction is then applied to form cell attached configuration creating a very tight 'giga ohm' seal;
- Further gentle suction allows cell membrane rupture whilst maintaining the seal and the internal contents of the cytosol can be manipulated by mixture with the internal solution from the patch pipette.

Cell preparation for imaging

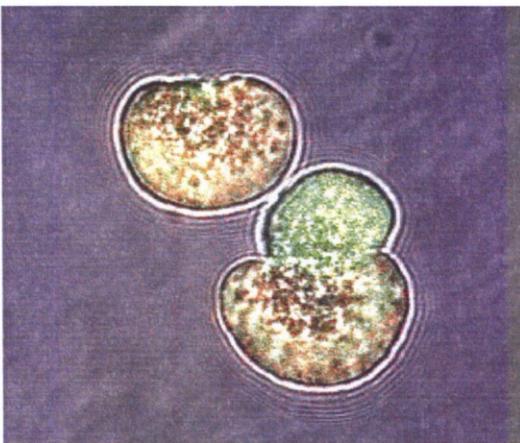
Fluorescent indicator loading

Pancreatic acinar cells once isolated were loaded with fluorescent indicators achieved by incubation with a membrane permeant form of the indicator. Appropriate volumes of fluorescent indicator were added to the 2ml cell suspension in standard extracellular solution and left for the required period of time at room temperature with gentle agitation to facilitate loading. The cells were then resuspended in a further 13mls of extracellular solution to ensure a good washout of the dye outside the cells and the Dimethyl Sulphoxide (DMSO) which the dyes are dissolved in and can be detrimental to the cells. Following the resuspension, the cells were spun at 1000 revolutions per minute for 1 minute and the pellet suspended in approximately 2 – 3mls of extracellular solution ready for use within 3- 4 hours of isolation. (See table 2.1 for a list of all fluorescent indicators used and the loading protocols).

The calcium indicators used throughout this study, Fluo – 4 and Fura – 2, were in the membrane permeant acetylmethoxy ester (AM) form (Molecular Probes/ Invitrogen, Eugene, OG, USA) and upon passage across the cell membrane are cleaved by intracellular esterases rendering them cell membrane impermeant. The fluorescent indicators are loaded predominantly into the cytosolic compartment of the cell (see table 2.1 for the exact loading procedures) and exhibit an increase in fluorescence upon binding to Ca^{2+} and therefore, increasing fluorescence, with an increase in cytosolic Ca^{2+} (figure 2.3).

Figure 2.3

Pancreatic acinar cells, as visualised with the Zeiss LSM confocal microscope. The cells are loaded with the Calcium sensitive dye, Fluo 4 (green). NADH autofluorescence is also visible (red) indicating the position of the mitochondria.



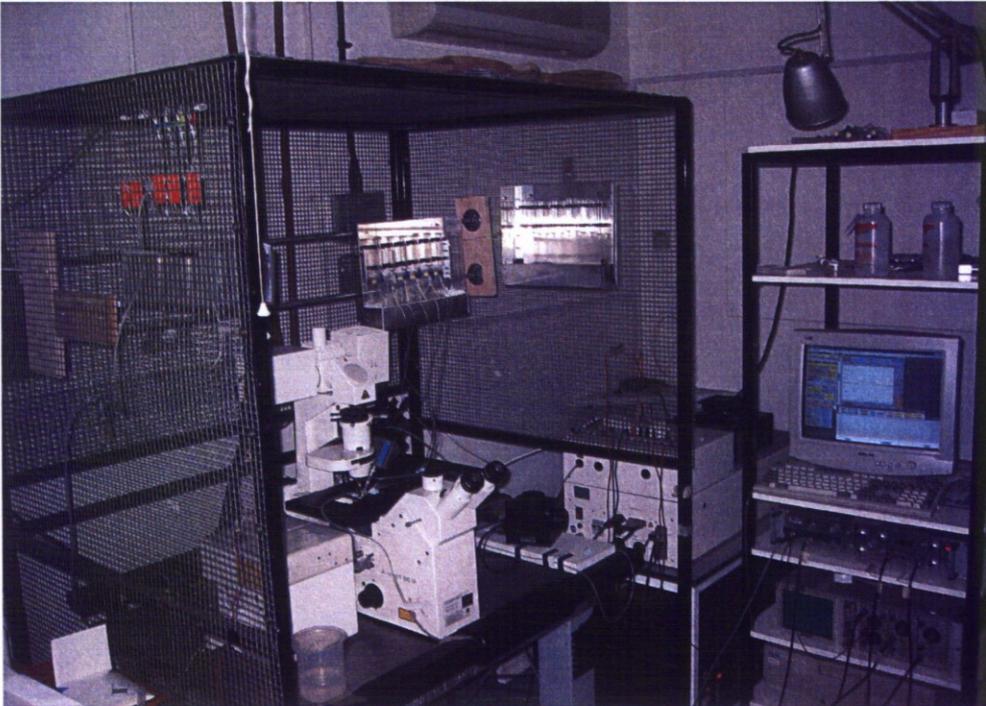
The top cell in the above doublet is exhibiting an increase in fluorescence due to an increase in cytosolic calcium

Perfusion systems

The exchange of bath solutions during experimentation is achieved via a gravity fed perfusion system. For all experiments a small volume of cell suspension was placed on a thin glass coverslip (BDH, 1mm) attached to a locally handmade Perspex perfusion chamber (total volume capacity approx. $100\mu\text{m}^2$). After a period of approximately three minutes to allow cells to attach spontaneously to the coverslip, perfusion with the extracellular solution is started. The perfusion system consists of six plastic syringes each fitted with a two-way valve so that the control of solution delivered to the bath, from each syringe, is maintained. Each two way tap valve is connected at the other end to thin bore silicon tubing which converges in a six way manifold into the perfusion chamber. A vacuum line is placed at the opposite side of the perfusion chamber to allow removal of the solutions flowing into the bath (figure 2.4 and 2.5).

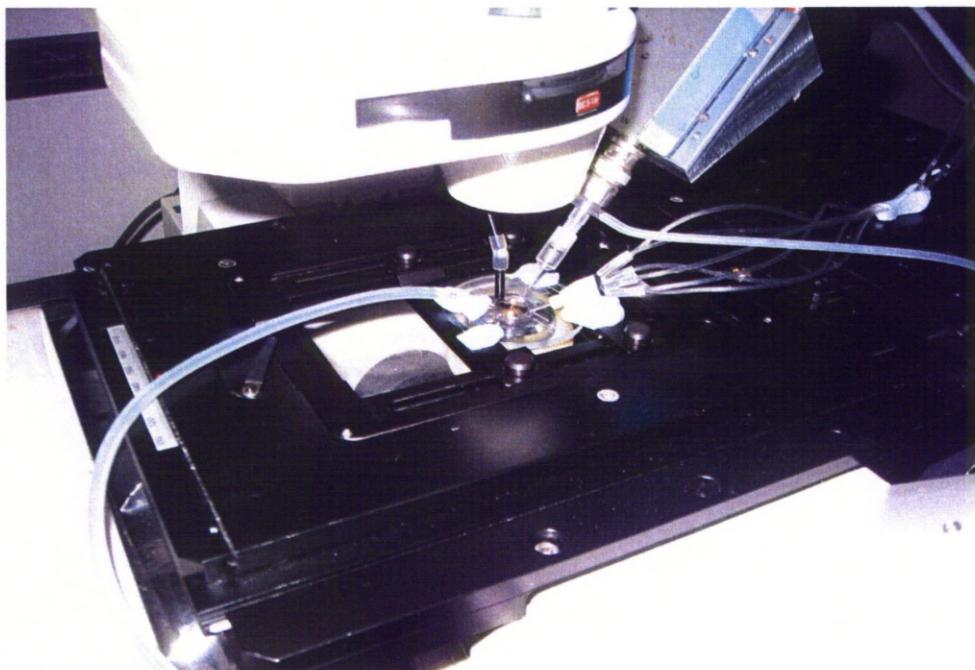
In certain experiments, certain chemicals were used in the external perfusion fluid (eg. Caffeine, BNPP, etc). Each particular situation will be explained as per experimental protocol in the specific chapter.

Figure 2.4



Photograph showing the Zeiss LSM 510 confocal microscope, housed in a faraday cage sitting on an air table to reduce vibrations. The perfusion system attached to the posterior wall of the cage. (Note the electrophysiological software to the right)

Figure 2.5



View on the microscope stage with the perfusion system shown.

The patch pipette holder is shown with a patch pipette going into the bath and attached to a cell. The tubing to the left is for gentle suction to prevent flooding of the bath.

Fluorescence measurements

Ca²⁺ measurements

Confocal imaging of cells loaded with fluorescent dyes was performed using a Zeiss LSM510 system (Carl Zeiss GmbH, Germany) to assess the effects of the bile salts TLC-3-S, the non-oxidative ethanol metabolite fatty acid ethyl esters POAEE (10 μ M dissolved in 10mM ethanol), the fatty acid POA (10 μ M) or differing concentrations of ethanol, on pancreatic acinar cell Ca²⁺ signalling induced by the different second messengers IP₃, cADPr and NAADP applied via the internal solution in the patch pipette (figure 2.5).

The Zeiss LSM 510 system (Carl Zeiss GmbH, Germany) was used to assess the Ca²⁺ signalling response of the secretagogues ACh (20-50nM) and CCK (1-10pM) with either the addition of the bile salts TLC-3-S (10 μ M) or the ethanol metabolites POAEE (10 μ M) together. Cells were loaded with Fluo-4 AM (2.5 μ M) or Fura Red (5 μ M) for 35mins at room temperature to measure [Ca²⁺]_c. Experiments were performed using the multi-track configuration of the Zeiss LSM software which allows sequential imaging using both an Argon laser and a UV laser. The fluorescence of Fluo-4 and Fura Red was excited using an Argon 488nm laser line with emission collected using a LP505nm filter. NADH autofluorescence used to measure mitochondrial metabolism was measured using a UV (351nm) excitation laser line and emission collected using a BP 385-470 filter. The images obtained consisted of 256 x 256 pixels, and the optical section chosen to be 5-6 μ M. A C-Apochromat x63 objective with a numerical aperture of 1.2 was used in all experiments and all images were analysed using Zeiss LSM software.

Mg²⁺ and indirect ATP measurement

Further experiments were performed to determine changes of intracellular ATP concentrations after loading with Mg Green-AM (4 μ M) for 30 min at room temperature. Since ATP has a ten-fold greater affinity for magnesium ions than ADP and most intracellular magnesium is present as MgATP, this dye monitors changes in ADP: ATP ratios that may indicate maintenance or depletion of intracellular ATP supplies, the latter demonstrated by an increase in Mg Green fluorescence (excitation 488 nm, emission 505–550 nm) as $[Mg^{2+}]_c$ rises in the presence of a calcium- and magnesium-free extracellular solution, or magnesium only free solution. Simultaneous NADH autofluorescence used to measure mitochondrial metabolism was again measured using a UV (351nm) excitation laser line and emission collected using a BP 385-470 filter. The images obtained consisted of 256 x 256 pixels, and the optical section chosen to be 5-6 μ M. A C-Apochromat x63 objective with a numerical aperture of 1.2 was used in all experiments and all images were analysed using Zeiss LSM software.

Cell toxicity measurement

In some experiments the effects of the bile acids Taurolithocholic – 3 – sulphate (TLC-3-S) and POAEE on physiological Ca²⁺ signalling was assessed by measuring whether the cells had undergone necrosis or not. This was achieved by the addition of propidium iodide (10 μ M) (Criddle et al, 2006) to the extracellular solution by micropipette injection so as to not disturb the high pipette-cell seal resistance. Images were taken at 0 and 10 minutes after the addition of the propidium iodide (Zeiss LSM; excitation 375nm, emission >400nm) to assess necrotic changes in both the patched cell and cells around it.

Table 2.1

The loading protocols of each fluorescent dye used

Fluorescent indicator	Loading Concentrat ⁿ	Loading temp(°C)	Loading time (mins)	Excitation (nm)	Emission (nm)	Target compartment
Fluo- 4 AM	3μM	36.5	30-35	488	500-550	Cytosolic Ca ²⁺
Fura- 2 AM	2μM	36.5	20-30	364	500-600	Cytosolic Ca ²⁺
Propidium iodide	10μM	Room (20)	10	375	>400	Nucleus
Magnesium Green AM	3μM	36.5	25-30	488	500-550	Cytosolic Mg ²⁺

RESULTS

Chapter 3

**Direct activation of cytosolic Ca²⁺ signalling and
enzyme secretion by cholecystokinin in human
pancreatic acinar cells**

Abstract

Background & Aims:

Cholecystokinin (CCK) has been thought to act only indirectly on human pancreatic acinar cells via vagal nerve stimulation, rather than by direct CCK receptor activation as demonstrated on rodent pancreatic acinar cells. Whether CCK (CCK-8 and human CCK-58) can act directly on human pancreatic acinar cells was tested.

Methods:

Human acinar cells were freshly isolated from pancreatic transection line samples, loaded with Fluo4-AM or quinacrine, and examined for Ca^{2+} , metabolic and secretory responses to CCK-8, human CCK-58 or acetylcholine (ACh) with confocal microscopy.

Results:

CCK-8 and human CCK-58 at physiological concentrations (1-20 pM) elicited rapid, robust, oscillatory rises of the cytosolic Ca^{2+} ion concentration ($[\text{Ca}^{2+}]_c$), showing apical to basal progression, in acinar cells from fourteen patients with unobstructed pancreata. The $[\text{Ca}^{2+}]_c$ rises were followed by increases in mitochondrial ATP production and secretion. CCK-elicited Ca^{2+} signals and exocytosis were not inhibited by atropine (1 μM) or tetrodotoxin (100 nM), demonstrating that CCK was unlikely to have acted via neurotransmitter release. CCK-elicited Ca^{2+} signals were inhibited completely and reversibly by caffeine (20

mM), indicating involvement of intracellular inositol trisphosphate receptor Ca^{2+} release channels. ACh (50 nM) elicited similar Ca^{2+} signals.

Conclusions:

CCK at physiological concentrations in the presence of atropine and tetrodotoxin elicits cytosolic Ca^{2+} signalling, activates mitochondrial function and stimulates enzyme secretion in isolated human pancreatic acinar cells. From this, the conclusions that may be drawn are that CCK acts directly on acinar cells in the human pancreas.

Introduction

The mechanism by which cholecystokinin (CCK) regulates human pancreatic function has not yet been clearly clarified, partly because of obvious difficulties in sampling normal pancreatic tissue from humans (Miller, 1996). Extensive studies of mouse and rat pancreatic acinar cells have shown that physiological concentrations (1-20 pM) of CCK, like acetylcholine (ACh), elicit cytosolic Ca^{2+} signals that control secretion (Petersen et al, 2008). Such signals result from a direct action of CCK on CCK receptors in the pancreatic acinar cell plasma membrane (Jensen et al, 1989), and *in vivo*, from vagal (neuronal) afferents stimulating acinar cell muscarinic receptors through vagal loops (Li et al, 1993). Failure to show functional responses to CCK in human pancreatic acinar cells (Ji et al, 2001), however, has led to the conclusion that CCK only stimulates human pancreatic acinar cells through vagal afferents (Ji et al, 2001, Owang et al, 2004, Dufresne et al, 2006).

Without cytosolic Ca^{2+} release via inositol trisphosphate receptors (IP_3Rs), CCK and ACh cannot stimulate pancreatic acinar cell secretion (Petersen et al, 2008, Futatsugi et al, 2005). As previously demonstrated, the cytosolic Ca^{2+} release elicited by ACh activates Ca^{2+} -dependent potassium channels and stimulates amylase secretion in human pancreatic tissue segments (Petersen et al, 1985). I sought to determine whether CCK (CCK-8 or human CCK-58) (Eysselein et al, 1990, 1990) at physiological concentrations can directly activate human pancreatic acinar cells. The experiments demonstrated here show that morphologically normal acinar cells immediately isolated from fresh samples of normal human pancreatic tissue produce robust, oscillatory

cytosolic Ca^{2+} signals in response to CCK-8 and human CCK-58 (up to 20 pM) as well as ACh (up to 50 nM) stimulation, resulting in an increase of mitochondrial metabolism and secretion. The actions of CCK were unaffected by atropine (1 μM) and tetrodotoxin (100 nM) and therefore were not mediated by ACh nor by action potential-mediated release of other neurotransmitters, which in theory might have been released from nerve endings possibly adhering to isolated pancreatic acinar cells.

Materials and Methods

Human Pancreatic Samples were collected as previously described in chapter 2 (see pages 121-124).

Isolation of pancreatic acinar cells

Single acinar cells and acinar cell clusters were isolated by a method adapted from rodent work (Criddle et al, 2006). Each pancreatic sample was injected at several points with collagenase (200 U/mL from Worthington Biochemical, Lakewood, NJ with 1 mM benzamidine or 0.01% soybean trypsin inhibitor, with 100nM tetrodotoxin, a potent neurotoxin which blocks diffusion of sodium through the sodium channel, thus preventing depolarization and propagation of action potentials in neurones). The tissue was finely minced and incubated at 37°C for up to 15 min, collagenase drained, and tissue suspended in extracellular solution. The suspension was triturated and supernatant collected, a process repeated until the supernatant was clear. Cells in the supernatant were pelleted by centrifugation at 1000 revs/min for 1 min, triturated again in fresh extracellular solution, filtered (70 µm Cell Strainer, BD Biosciences, Bedford, MA) to remove undispersed tissue, and centrifuged exactly as before. The final pellet of dispersed cells was re-suspended in extracellular solution and all experiments performed at room temperature within 3 hours of isolation.

Confocal imaging

Confocal imaging of cells loaded with fluorescent dyes was undertaken using a Zeiss LSM510 system (Carl Zeiss, Jena, Germany). Fluo 4-AM (3 μM) was loaded for 30 min to measure changes in $[\text{Ca}^{2+}]_c$ in response to physiologically representative concentrations of CCK-8 or CCK-58 (both 1-20pM) or ACh (50nM). The LSM510 multi-track configuration was used for simultaneous measurement of $[\text{Ca}^{2+}]_c$ (excitation 488 nm; emission >510 nm) and reduced nicotinic adenine dinucleotide (NADH) autofluorescence (excitation 351 nm; emission 385-470 nm) to assess mitochondrial metabolism (Hajnoczky et al, 1995, Voronina et al, 2002). Exocytotic responses were measured using quinacrine (10 μM), loaded at 37°C for 10 min, which selectively stains the acidic compartments and has been used to quantify exocytosis from a variety of cell types including rodent pancreatic acinar cells in response to ACh (excitation 488 nm; emission >505 nm) (Pralong et al, 1990, Campos-Toimil et al, 2000, Park et al, 2004). In some experiments rhodamine 110, bis-(CBZ-L-isoleucyl-L-prolyl-L-arginine amide) dihydrochloride (BZiPAR), a cell permeant substrate for trypsin that becomes fluorescent after cleavage by the protease (excitation 488nm, emission $>505\text{nm}$) (Kruger et al, 2000, Raraty et al, 2000), was used to measure intracellular trypsin activity. Images were composed of 256 x 256 pixels, and optical sections were 5-6 μm . A C-Apochromat x 63 objective with a numerical aperture of 1.2 was used and image analysis completed with LSM510 software.

Amylase measurements

Amylase measurements were performed using two methods. Firstly, an on-line fluorometric assay system was used to measure amylase secretion, as previously described (Petersen et al, 1985). Briefly, small segments of human pancreas (total weight 50-100 mg) were placed in a Perspex flow chamber (1 ml volume) and superfused with physiological saline solution (1 ml/min) at 37°C. After a period of equilibration, tissues were stimulated with CCK (10 pM) and amylase secretion measured fluorometrically (excitation 485 nm; emission 520 nm) using amylase substrate (DQ starch from corn, BODIPY FL conjugate (100 ug/ml); EnzChek Ultra Amylase Assay Kit, Molecular Probes, Eugene, OR) and secretion expressed as units per min per mg tissue. In the present study α -amylase was used as a standard for calibration. Additional measurements were performed using spectrophotometric analysis of amylase secretion from freshly dispersed cells. Human pancreatic acinar cell suspensions were incubated with CCK (10 pM–10 nM) for 30 min following which amylase activity was measured by adding amylase substrate (Starch dyed with Remazol Brilliant Blue R; Sigma-Aldrich, Gillingham, UK) to the supernatant in a cuvette and fluorescence measured spectrophotometrically (emission 590 nm). Measurements were performed in triplicate and secretion expressed as a percentage of total amylase.

Solutions and chemicals

The extracellular solution contained (mM): 140 NaCl, 4.7 KCl, 1.13 MgCl₂, 1 CaCl₂, 10 D-glucose, 10 HEPES (adjusted to pH 7.35 with NaOH). Protease inhibitor tablets (one per 10 mL, Roche Diagnostics, Mannheim, Germany) and benzamidine hydrochloride (1

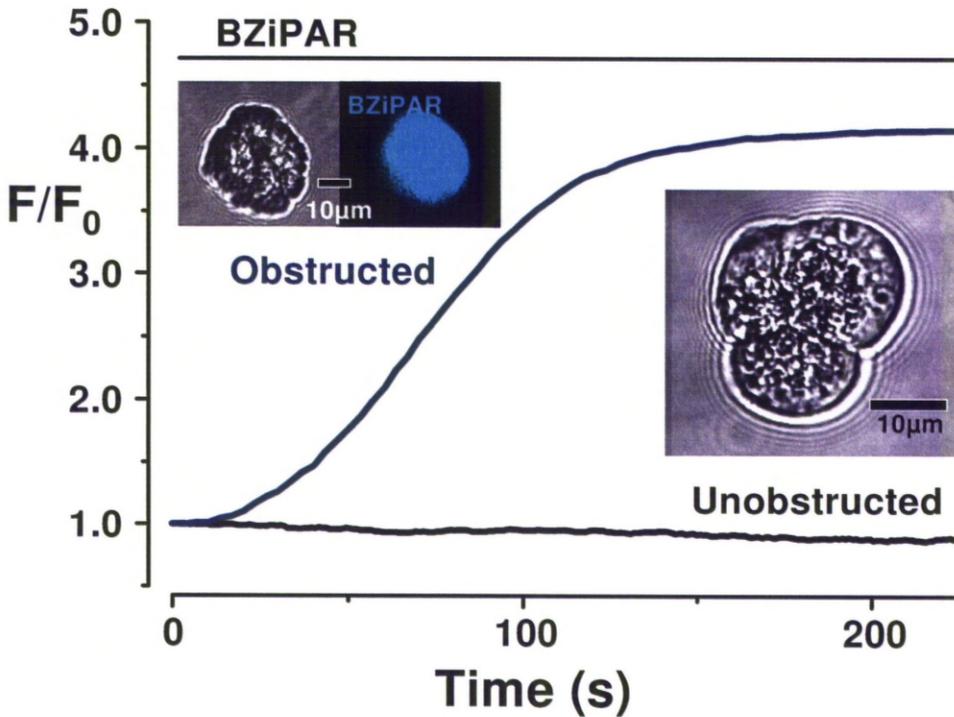
mM, Sigma-Aldrich) were added to limit damage before cell isolation. In some experiments tetrodotoxin (100 nM, Tocris Bioscience, Bristol, UK) was added to all solutions throughout, to prevent action potentials that could induce neurotransmitter release from any nerve endings that might have been adherent to isolated cells. Latterly pyruvate (100 μ M) was also added to boost substrate supply for mitochondrial metabolism. Fluo-4 AM and BZIPAR were from Molecular Probes. Human synthetic sulfated CCK-58 was obtained from UCLA Peptide Synthesis Facility (Dr. J. R. Reeve, Jr., Director). The human CCK-58 was synthesized using an Applied Biosystems Peptide Synthesizer (Foster City, CA, USA), unblocked and purified to >90% as described for the synthesis of rat CCK-58 (Reeve et al, 2004). Other chemicals were from Sigma-Aldrich of the highest grade available.

Results

Characteristics of patients, pancreatic samples and acinar cell isolates

Pancreatic samples were donated by fourteen patients (mean age 59 years; range 25-82) undergoing surgery between January 2004 to February 2008 (Table 3.1). Histopathological examination of the excised specimen at the pancreatic transection line confirmed normal pancreatic histology in every case, but also mild fibrosis in four. Isolated acinar cells typically showed excellent morphology, with strong polarity delineated by a distinct granular region in the apical pole of each cell, and no intracellular trypsin activity detectable with BZIPAR (Figure 3.1). Such cells were unlike those isolated in preliminary experiments from several obstructed pancreata, cells that showed indistinct morphology with little if any polarity, global intracellular trypsin activity (Figure 3.1) and no responses to secretagogues. Histopathological examination of the obstructed pancreata showed significant fibrosis in every case, accounting for an increased digestion time necessary to isolate these cells, and their evident injury excluding them from further use in these experiments (Table 3.2) (Figure 3.2).

Figure 3.1



Morphology and integrity of isolated human pancreatic acinar cell clusters loaded with 10µM BZiPAR, which fluoresces after trypsin cleavage of two side chains. Baseline BZiPAR trace and accompanying inset shows no detectable trypsin activity and typical, well-preserved morphology with apical arrangement of granules (facing centrally towards original acinar lumen) of an isolated acinar cell cluster obtained from a sample of an unobstructed pancreas, as used in all subsequent experiments. Elevated BZiPAR trace and inset display trypsin activity in cells with poorly defined morphology, typical of an acinar cell cluster isolated from an obstructed pancreas, showing injury from the prolonged isolation procedure with excessive collagenase digestion. BZiPAR fluorescence data are given as normalised changes from basal values (F/F_0).

Table 3.1

Characteristics of patients, surgical procedures and pancreata from which acinar cells were isolated that demonstrated CCK- and ACh-elicited Ca²⁺ signaling, mitochondrial responses and exocytotic secretion.

Age	Sex	Surgical procedure^a	Final diagnosis^b	Main duct^c	Pancreas^d
57	F	Pancreatoduodenectomy	IPMN ^e	Normal	No fibrosis
58	F	Pancreatoduodenectomy	Duodenal adenoma	Normal	No fibrosis
59	M	Pancreatoduodenectomy	Simple squamous cyst	Normal	No fibrosis
66	M	Pancreatoduodenectomy	Ampullary adenoma	Normal	Mild fibrosis
68	M	Pancreatoduodenectomy	Ampullary carcinoma	Normal	Mild fibrosis
71	F	Enucleation ^f	Insulinoma	Normal	No fibrosis
25	F	Left pancreatectomy	Gastrinoma	Normal	Mild fibrosis
27	M	Left pancreatectomy	Serous cystadenoma	Normal	No fibrosis
55	M	Left pancreatectomy ^g	Endocrine carcinoma	Normal	No fibrosis
58	F	Left pancreatectomy ^g	Adenosquamous carcinoma	Normal	Mild fibrosis
59	F	Left pancreatectomy	Mucinous cystadenoma	Normal	No fibrosis
65	F	Left pancreatectomy	Serous cystadenoma	Normal	No fibrosis
72	F	Left pancreatectomy	Serous cystadenoma	Normal	No fibrosis
82	F	Left pancreatectomy	Serous cystadenoma	Normal	No fibrosis

^aPancreatoduodenectomy was pylorus-preserving Kausch-Whipple resection

^bHistology of resected specimen

^cData from Computerised Tomography and/or histology

^dHistology of non-neoplastic pancreatic parenchyma at transection line in resected specimen

^eIntraductal papillary mucinous neoplasm

^fPancreatic sample taken from normal parenchyma near to, but not contiguous with, completely excised insulinoma

^gWith splenectomy

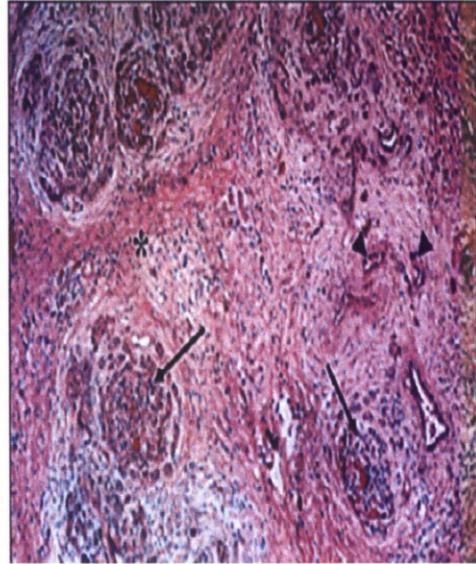
Figure 3.2

Histological slides of normal and fibrotic human pancreas

Normal Pancreas



Fibrotic Pancreas



Standard haematoxylin and eosin (H+E) staining of the normal human pancreas (left) and the fibrotic pancreas (right), as encountered during the early phase of experimentation. Note, on the right, the broad bands of fibrosis (asterisk), some residual acinar tissue (arrows), an inflammatory cell infiltrate and distorted ducts.

CCK-8 and human CCK-58 at physiological concentrations elicit cytosolic Ca²⁺ signals in human pancreatic acinar cells

CCK-8 (1-20 pM) elicited clear elevations of $[Ca^{2+}]_C$ from a stable basal level that included oscillatory responses (Figure 3.3; total number of responding cells = 63). Since nerve endings could in principle adhere to isolated cells, most experiments were done with the muscarinic receptor antagonist atropine (1 μ M), which blocks intracellular Ca²⁺ release and all Ca²⁺-mediated effects in response to electrical nerve stimulation or direct application of ACh, but not application of CCK, in rodent acinar cells (Pearson et al, 1981, Petersen et al, 1992). To exclude effects of non-cholinergic neurotransmitters, some experiments were also conducted during continuous application of both atropine (1 μ M) and tetrodotoxin (100 nM), which blocks voltage-gated Na⁺ channels and thus action potentials, inhibiting neurotransmitter release (Figure 3.3A). The rises of $[Ca^{2+}]_C$ elicited by CCK-8 were not inhibited by atropine (Figure 3.3B; n=25) nor atropine plus tetrodotoxin (n=28), demonstrating that the effects of CCK-8 were not caused by ACh or other neurotransmitters released from adherent nerve endings. As expected from early work (Petersen et al, 1985), ACh itself, at a low quasi-physiological concentration (50 nM), evoked a clear increase in $[Ca^{2+}]_C$ of a similar magnitude to increases evoked by CCK-8, including oscillatory patterns (Figure 3.4A; n=27); no Ca²⁺ responses occurred in response to ACh in the presence of atropine (n=0/10).

Figure 3.3

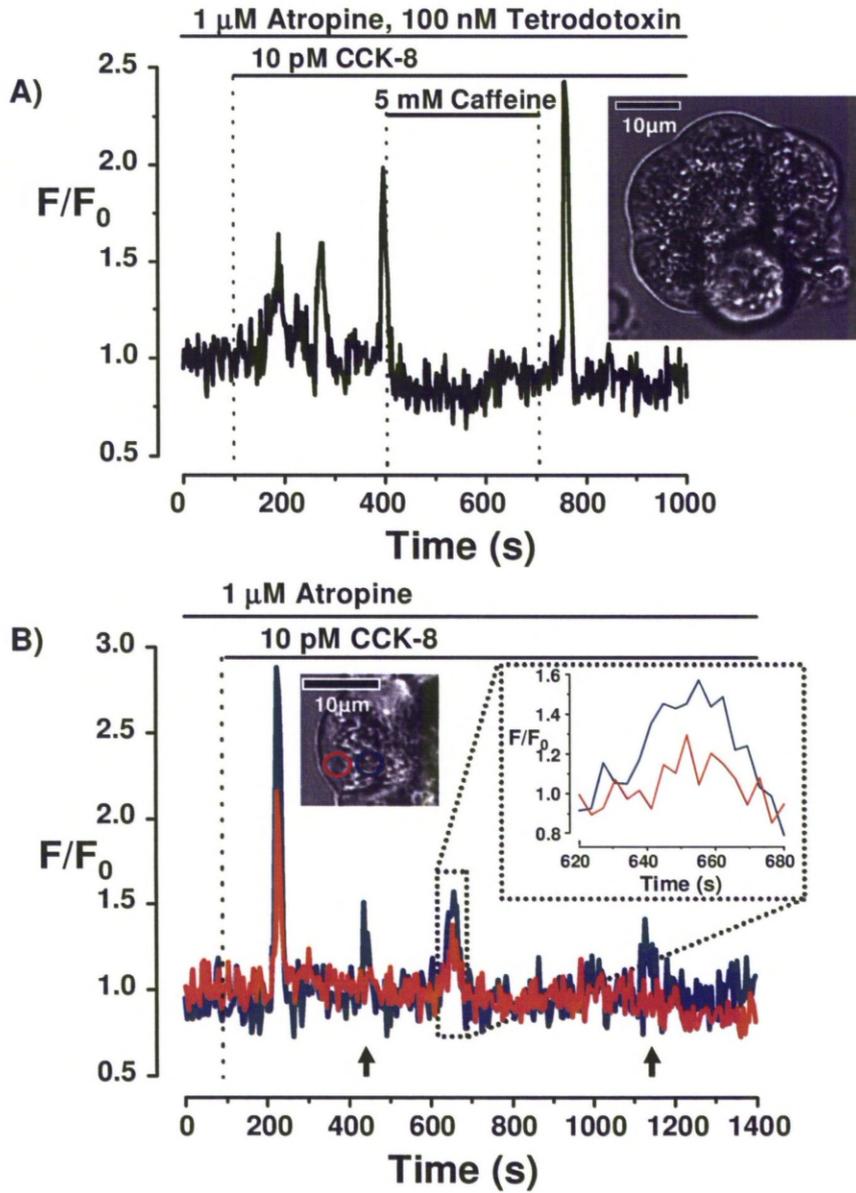


Figure 3.3

Representative responses of $[Ca^{2+}]_C$ in human pancreatic acinar cells isolated from fourteen unobstructed pancreata.

A) CCK-8 at a representative low physiological concentration (10 pM; responses were obtained with 1-20 pM CCK-8 from a total of 63 cells) evoked oscillatory responses within cells in cluster (*inset*, recording from single cell) in the presence of both atropine and tetrodotoxin. 5mM caffeine completely and reversibly blocked the elevations of $[Ca^{2+}]_C$ (observed in 15 cells during application of up to 20 pM CCK-8, and 11 cells during application of up to 20 pM human CCK-58). Data are given as normalised changes in fluorescence from basal values (F/F_0).

B) Apical (*blue circle in inset*) and basolateral (*red circle*) recordings of $[Ca^{2+}]_C$ responses to 10 pM CCK-8 in the presence of atropine, showing four apical spikes that, as in rodent cells, progress into the basolateral region on some occasions (absence of basal progression marked by arrows). Inset shows apical to basal delay of $[Ca^{2+}]_C$ response progressing into the basolateral region; data are given as normalised changes in fluorescence from basal values (F/F_0)

Figure 3.4

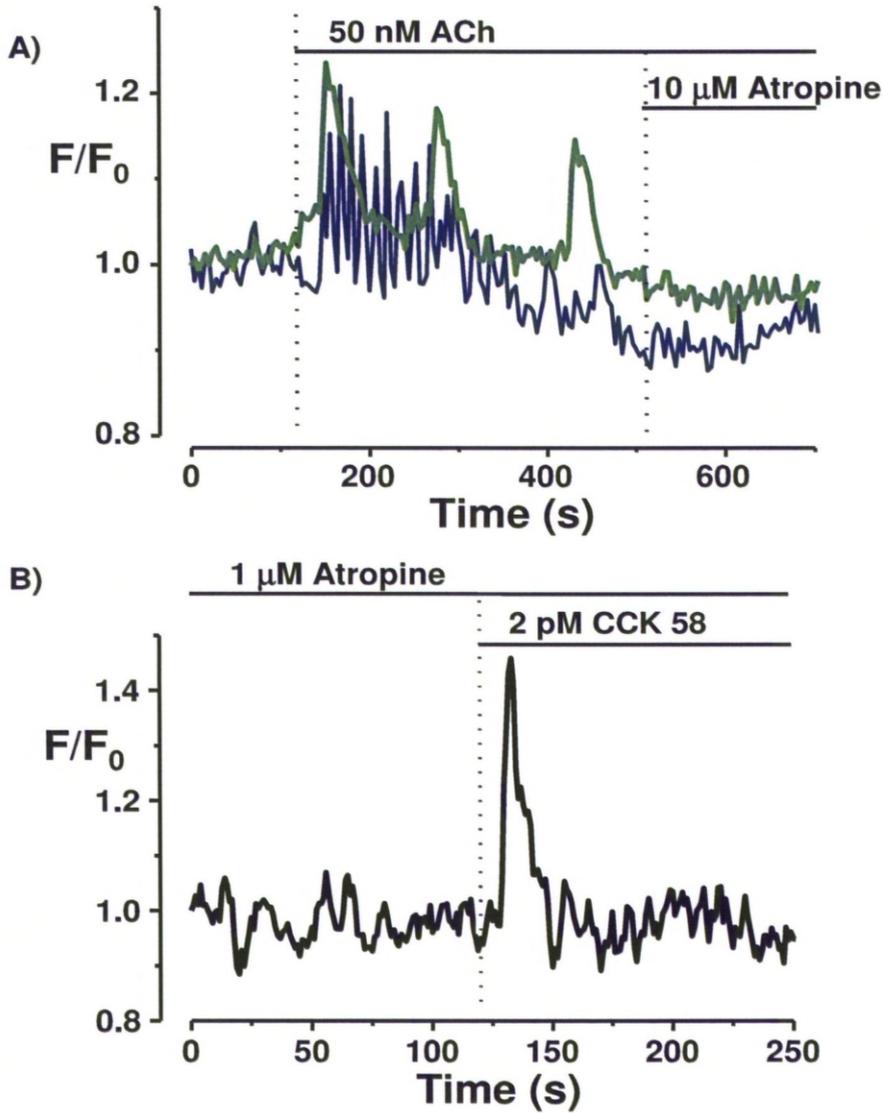


Figure 3.4

Representative Ca^{2+} signaling responses of isolated human pancreatic acinar cells elicited by 50 nM ACh and CCK-58.

A) Frequent, rapid, apical oscillations are seen in one cell (*blue trace*) while more prolonged, global transients are seen in the other (*green trace*), patterns identical to those seen in rodent pancreatic acinar cells. Atropine (10 μM) inhibits all further Ca^{2+} signaling responses.

B) Example of $[\text{Ca}^{2+}]_c$ response in isolated human pancreatic acinar cell following application of 2 pM human CCK-58 (responses obtained with 1-20 pM CCK-58 from a total of 19 cells, similar to responses elicited by CCK-8). Human CCK-58 was applied in the presence of atropine to prevent $[\text{Ca}^{2+}]_c$ signals being elicited by ACh that could have been released from adherent nerve endings activated by neural CCK receptors. All data are given as normalised changes in fluorescence from basal values (F/F_0).

CCK-8 and CCK-58 elicit Ca^{2+} signals which are of similar amplitude to those generated by ACh.

With the uncertainty that surrounds the physiological action of CCK directly on the pancreatic acinar cell of the hormones used (Owang et al, 2004, Reeve et al, 2003) it is important to ascertain whether any Ca^{2+} signals generated are similar, or not, to those previously observed in different (rodent) cell types.

Because CCK-58 is the predominant, endogenous, circulating form of CCK in humans, and has a unique and specific 58 amino acid sequence (Eysselein et al, 1990), human CCK-58 might display differences in activity from the widely used CCK-8 (Reeve et al, 2003, Kreis et al, 1997, Yamamoto et al, 2005). Therefore, isolated human pancreatic acinar cells were also tested for responses to human CCK-58. As with CCK-8, physiological concentrations of human CCK-58 (up to 20 pM) elicited clear rises of $[\text{Ca}^{2+}]_c$ that were not inhibited by atropine (Figure 3.4B; n=19), again of similar magnitude to those induced by ACh.

We measured the magnitude of Ca^{2+} fluorescence increase from baseline to peak in response to each secretagogue and found no significant difference in response in Ca^{2+} fluorescence observed between each stimulant tested (Figure 3.5).

Figure 3.5

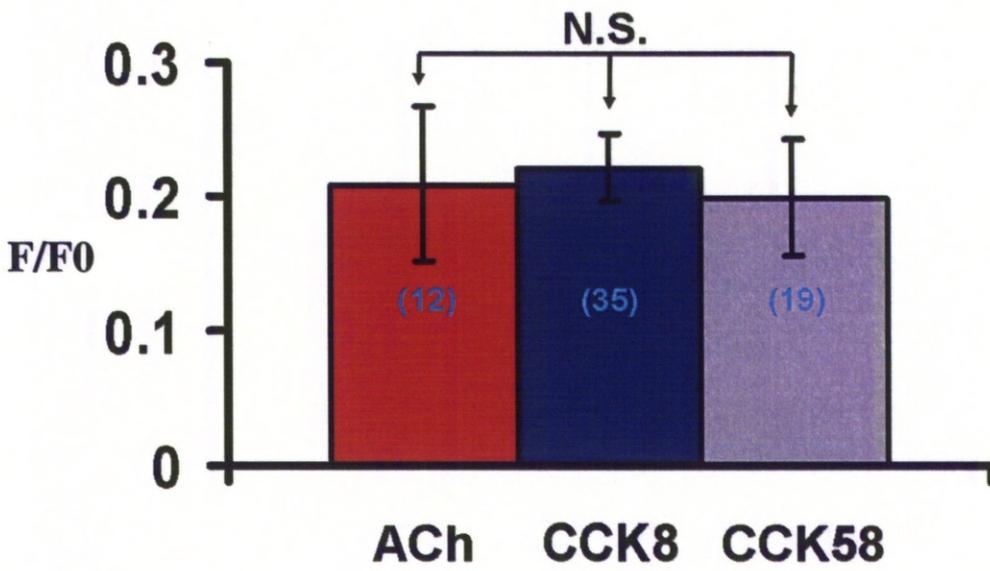


Figure 3.5

Comparative plot showing similarity of maximum amplitude of $[Ca^{2+}]_c$ responses elicited by ACh, CCK 8 and CCK58. Note there is no statistical difference between the Ca^{2+} signals generated in response to each of the physiological stimuli.

In rodent pancreatic acinar cells both ACh- and CCK-elicited cytosolic Ca^{2+} signals are generated primarily by Ca^{2+} release from the endoplasmic reticulum via IP_3R and ryanodine receptor Ca^{2+} channels (Petersen et al , 1992, 2008, Rizzuto et al, 2006). Since caffeine has been well characterized as a membrane-permeable inhibitor of IP_3Rs that inhibits both ACh- and CCK-elicited Ca^{2+} signals in rodent acinar cells (Petersen et al, 2008, Criddle et al, 2006, Petersen et al , 2006), I tested the effects of caffeine on the action of CCK in human cells. Caffeine (20 mM) reversibly abolished elevations of $[\text{Ca}^{2+}]_c$ elicited by CCK-8 (n=15) and by human CCK-58 (n=11); cessation of caffeine administration was followed by prompt reversal of inhibition (as illustrated during CCK-8 stimulation in Figure 3.3A and later in Figure 3.8).

High concentrations of CCK induce global Ca^{2+} transients in rodent acinar cells that are prolonged (Raraty et al, 2000), and followed by an elevated plateau of $[\text{Ca}^{2+}]_c$ as long as the supra-maximal stimulus is continued. We applied high concentrations of CCK-8 (5-10 nM) to isolated human pancreatic acinar cells in the presence of atropine and tetrodotoxin, and observed prolonged, global Ca^{2+} transients followed by an elevated plateau of $[\text{Ca}^{2+}]_c$, which returned to baseline when the stimulus was stopped (Figure 3.6; n=15).

Figure 3.6

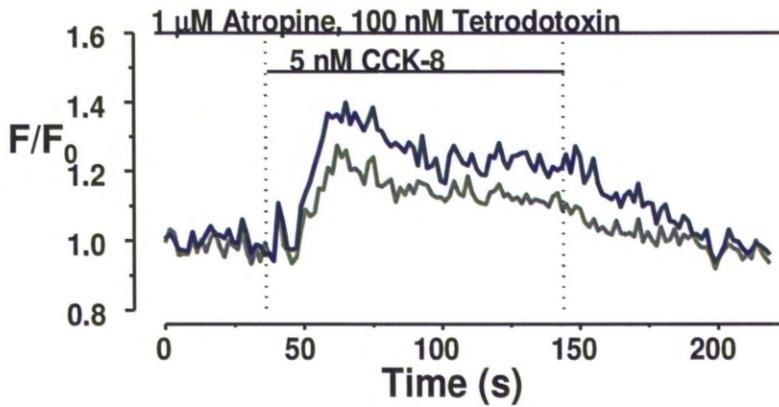


Figure 3.6

Example of changes in $[\text{Ca}^{2+}]_c$ within two isolated human pancreatic acinar cells during hyperstimulation with 5 nM CCK-8, showing a pattern similar to that in rodents and implicated in pancreatic acinar cell injury from prolonged hyperstimulation. An initial, large, prolonged, global rise in $[\text{Ca}^{2+}]_c$ occurred in both cells, that fell to a plateau level as long as stimulation was continued. Upon cessation of the stimulus, $[\text{Ca}^{2+}]_c$ returned to baseline levels. Fluorescence was normalised from basal values (F/F_0).

CCK-elicited Ca²⁺ signals in human pancreatic acinar cells start in the apical pole and progress to the basolateral region

Ca²⁺ signals in rodent pancreatic acinar cells always occur locally in the apical (granular) pole due to the concentration of IP₃Rs in this region (Petersen et al, 2008, Nathanson et al, 1994), and may progress globally as waves into the basolateral region (Petersen et al, 2008, Nathanson et al, 1994). Figure 3.3B shows that in human pancreatic acinar cells, both local and global Ca²⁺ signals occur. In the human pancreatic acinar cells, subcellular region of interest analysis demonstrated that CCK (both CCK-8 and human CCK-58) as well as ACh initiated Ca²⁺ release in the apical pole, and that when global Ca²⁺ signals occurred, these spread from the apical into the basolateral region of the cells (Figure 3.7 with CCK-8 and ACh).

Figure 3.7

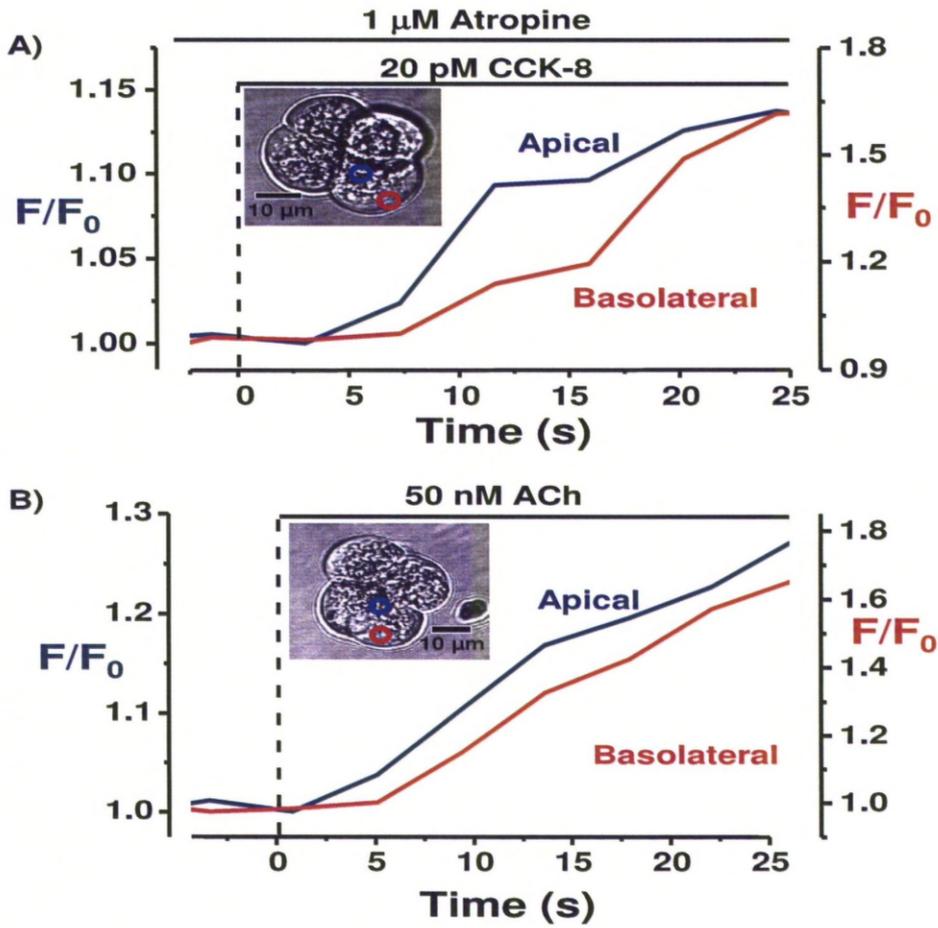


Figure 3.7

Spatio-temporal characteristics of global $[Ca^{2+}]_C$ rises in human pancreatic acinar cells in response to CCK and ACh stimulation. Insets show light-transmitted images of several responding cells, one marked with apical (*blue circle*) and basal (*red circle*) regions of interest from which Fluo-4 fluorescence intensity (normalised changes from basal values, F/F_0) was simultaneously recorded.

A) Graph of Fluo-4 fluorescence changes in two regions of the cell elicited by 20 pM CCK-8, showing characteristic initiation of the $[Ca^{2+}]_C$ rise in the apical region, subsequently spreading into the basal region (also seen in cells stimulated by human CCK-58).

B) Graph of Fluo-4 fluorescence showing similar changes to that in **(A)** in response to 50 nM ACh, again demonstrating fast apical to basal spread of the $[Ca^{2+}]_C$ wave.

Ca²⁺ signals elicited by CCK-8 and human CCK-58 are followed by an increase in mitochondrial NADH concentrations

Acinar cell fluid and enzyme secretion are energy-dependent processes, tightly controlled by Ca²⁺ signalling and mitochondria tailoring ATP production to the increased needs of the cell when secreting (Petersen et al, 2008, Rizutto et al, 2006). This increase in mitochondrial energy delivery can be monitored by the rise of the mitochondrial NADH concentration, which has been assessed in mouse pancreatic acinar cells by measuring mitochondrial autofluorescence (Hajnoczky, 1995, Voronina et al, 2002, Pralong et al, 1990), at specific excitation and emission spectra different from those of the Ca²⁺ dye (Fluo 4) used here. In the human cells, the caffeine-sensitive rises of [Ca²⁺]_c elicited by CCK-8 and CCK-58 were followed by an increase in NADH concentration; exemplified by responses to CCK-8 in Figure 3.8B. Secretagogue-elicited rises in NADH autofluorescence were observed following [Ca²⁺]_c signals in all cells examined (CCK-8: n=16; CCK-58: n=5; ACh: n=4).

Figure 3.8

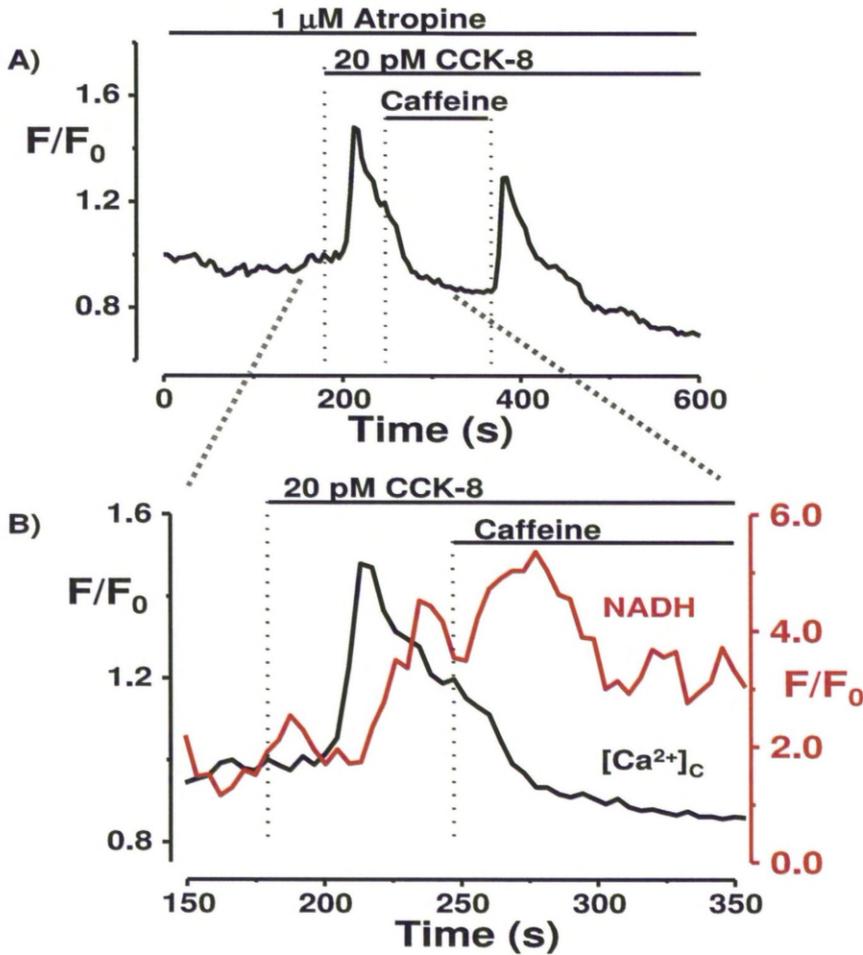


Figure 3.8 Mitochondrial NADH autofluorescence is increased in response to CCK-elicited $[\text{Ca}^{2+}]_c$ elevations (normalised from basal values, F/F_0). **A)** Rapid increase of $[\text{Ca}^{2+}]_c$ elicited by 20 pM CCK-8 is reversibly inhibited by 20 mM caffeine.

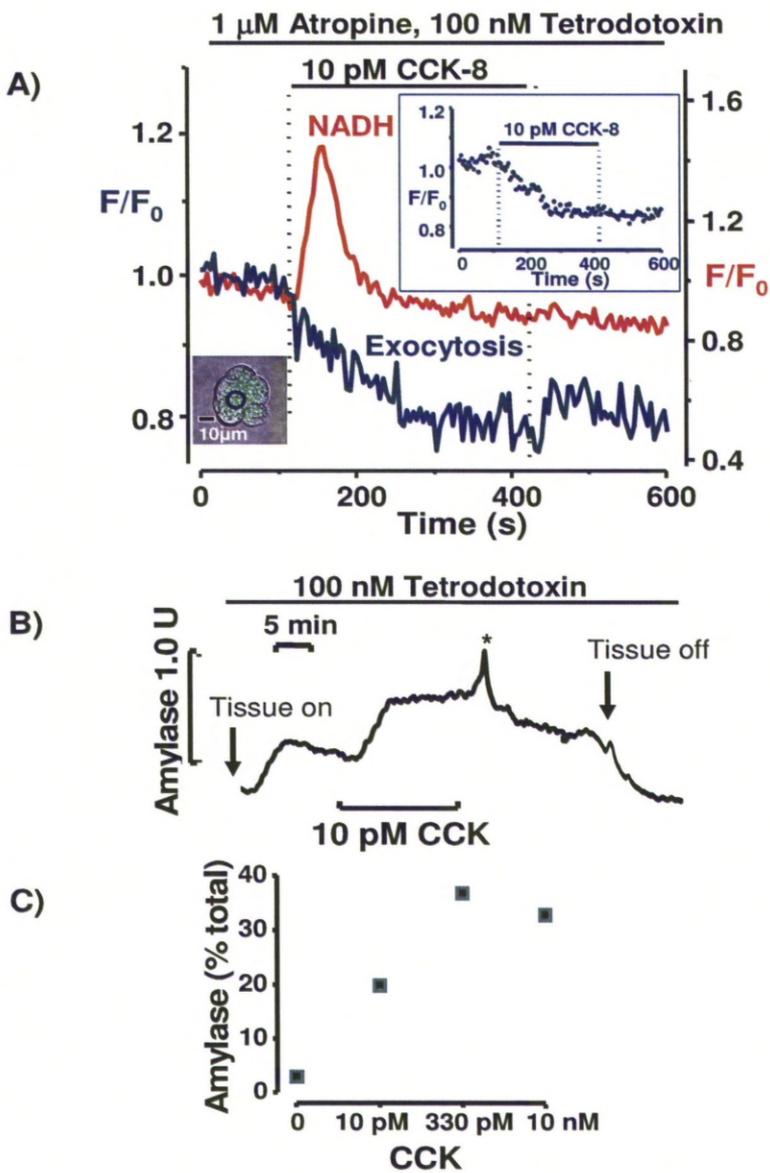
B) $[\text{Ca}^{2+}]_c$ rise is followed by a rise of NADH autofluorescence (*red trace*, normalised from basal values, F/F_0). Similar changes were seen in response to all secretagogues tested (CCK-8, CCK-58 and ACh).

CCK-8 evokes a coordinated increase of mitochondrial NADH and secretion from human pancreatic acinar cells

Quinacrine is an acidophilic fluorescent dye preferentially taken up by secretory granules that has been widely used to detect and quantify exocytosis from pancreatic acinar cells (Campos-Toimil et al, 2000, Park et al, 2004). The excitation and emission spectra required, however, prevent simultaneous measurement of Ca^{2+} responses using standard fluorescent dyes. During stimulation for ~5 min, physiologically representative concentrations of ACh have been shown to evoke secretion of 10-15% of the 200-300 zymogen granules within each intact isolated rodent pancreatic acinar cell (Campos-Toimil, 2000), which has been resolved at the level of individual exocytotic events with quinacrine (Park et al, 2004). We used quinacrine to examine the secretory function of isolated human pancreatic acinar cells during stimulation with 10 pM CCK-8, in the presence of atropine and tetrodotoxin, and observed exocytosis of a zymogen granule pool similar in rate and magnitude to that previously observed in such cells during ACh stimulation. The exocytotic secretion was accompanied by an elevation in mitochondrial NADH generation, indicative of a coordinated increase in mitochondrial ATP production (Figure 3.9A) (Petersen et al, 2008, Hajnoczky et al 1995, Voronina et al, 2002, Pralong et al, 1990). The rate and magnitude of the change in quinacrine fluorescence was similar to that seen in murine pancreatic acinar cells, replicated in 7 cells in the presence of atropine and tetrodotoxin (Figure 3.9A inset). Responses to hyperstimulation with 10 nM CCK-8 were also examined in the presence of atropine and tetrodotoxin, and while hyperstimulation induced a prolonged increase in NADH autofluorescence, secretory responses were not as pronounced (replicated in 4 cells).

This finding is in keeping with the reduction in secretion seen in rodent pancreatic acinar cells following hyperstimulation, relative to the responses seen following stimulation with physiological concentrations of secretagogues (Saluja et al, 2007). Amylase secretion was measured directly using two conventional methods; an on-line fluorometric assay using segments of human pancreatic tissue, as reported previously (Petersen et al, 1985), and a cuvette-based spectrophotometric assay employing freshly dispersed pancreatic acinar cells. In the on-line assay, a physiological concentration (10 pM) of CCK-8 induced a sustained and reversible increase in amylase secretion from a basal value of 0.47 U/min per 100 mg of tissue to 0.93 U/min per 100 mg of tissue, an approximate doubling of the basal secretion rate (Figure 3.9B), much the same as that observed previously in human pancreatic tissue with ACh (Peterson et al, 1985). Similar responses were seen in the presence of tetrodotoxin and atropine from segments of a further four pancreata. Furthermore, spectrophotometric measurements from the supernatant of freshly dispersed cells demonstrated that CCK-8 (10 pM - 10 nM) elicited significant, concentration-dependent increases in amylase secretion, reaching a maximal amylase output of approximately 35% of total amylase (Figure 3.9C), that was reduced following hyperstimulation with 10 nM CCK. The same concentration-dependent spectrophotometric data were obtained in the presence of tetrodotoxin and atropine from the supernatant of freshly dispersed cells isolated from a further five human pancreata.

Figure 3.9 Demonstration of isolated human pancreatic acinar cell exocytotic secretory responses to CCK.



A) Exocytosis occurred in response to a representative physiological concentration of CCK-8 (10 pM) in the presence of atropine (1 μ M) and tetrodotoxin (100 nM), to prevent

neurotransmitter release or effects that might theoretically occur from possible adherent nerve endings. Typical quinacrine staining of zymogen granules is shown within the inset, used to measure exocytosis from the region of interest (*blue circle*). Upon stimulation, quinacrine fluorescence promptly fell from a steady baseline, reflecting loss of quinacrine-containing zymogen granules; not observed without stimulation. The responses were similar to those observed using quinacrine in isolated rodent pancreatic acinar cells. A coordinated increase in NADH autofluorescence occurred at the start of stimulation, indicative of increased ATP production to fuel secretion. Top right inset shows mean \pm sem exocytotic responses from seven cells in separate experiments with 10 pM CCK-8, all obtained in the presence of atropine (1 μ M) and tetrodotoxin (100 nM). All fluorescence changes were normalised from basal values (F/F_0).

B) Amylase secretion from superfused segments of human pancreatic tissue in the presence of tetrodotoxin (100 nM), monitored fluorometrically in the flow cell effluent. The basal amylase output, observed when the tissue is on-line (arrow), and reversible stimulatory effect of physiological (10 pM) CCK-8 are shown (*denotes air bubble through system); similar data were obtained from segments of a further four human pancreata.

C) Concentration-dependent amylase secretion, expressed as a % of total amylase, evoked from freshly isolated human pancreatic acinar cells by stimulation with CCK-8 (10 pM–10 nM), in the presence of atropine (1 μ M) and tetrodotoxin (100 nM). All measurements were performed in triplicate; identical, concentration-dependent responses were obtained from human pancreatic acinar cells isolated from a further five human pancreata.

Discussion

This data demonstrates convincingly for the first time that two different forms of CCK, CCK-8 and human CCK-58, activate human pancreatic acinar cells at a range of CCK concentrations normally expected *in vivo* (Walsh, 1978), generating robust and reversible intracellular Ca^{2+} signals that are followed by stimulation of mitochondrial metabolism and exocytosis of digestive enzymes. Pancreatic enzyme secretion occurs by exocytosis and is initiated by a rise in $[\text{Ca}^{2+}]_c$ (Petersen et al, 2008, 1985) that requires ATP (Petersen et al, 2008, Baker et al, 1978, Maruyama et al, 1993). It is therefore now clear that CCK acting on human pancreatic acinar cells can generate the Ca^{2+} signal that initiates secretion and also activates mitochondrial metabolism, producing the ATP needed to fuel the secretory process. Measurements of exocytosis and amylase secretion, carried out both in tissue segments and dispersed cells, confirm that functional CCK receptors are present in human pancreatic acinar cells, the stimulation of which leads to enzyme secretion.

It was demonstrated some years ago that ACh can elicit Ca^{2+} signals in human pancreatic acinar cells (Petersen et al, 1985). The effects of CCK described here, are not mediated by ACh, since the CCK-elicited Ca^{2+} signals were unaffected by the blockade with atropine as well as tetrodotoxin. The hypothesis most consistent with the new data is therefore that CCK acts directly on CCK receptors in the human pancreatic acinar cell plasma membrane. Nevertheless in the intact human body CCK may also activate sensory nerve endings and, via vagal loops, cause release of ACh in the pancreas, as in rodents (Li et al, 1993, Owang et al, 2004, Dufresne et al, 2006).

CCK is secreted by I cells in the mucosa of the small intestine (Liddel et al, 1997). There is debate about exactly which form of CCK is present in blood (CCK-58, CCK-33, CCK-22 or CCK-8), but they all share the same C-terminal heptapeptide amide sequence (-Tyr(SO₃)-Met-Gly-Trp-Met-Asp-PheNH₂) that is necessary for potent receptor binding (Dufresne et al, 2006). Substantial data indicate that human CCK-58 is the predominant circulating form in several species (Eysselein et al, 1990, 1990, Sun et al, 1992, Reeve et al, 2003), but despite the tertiary structure of its C-terminal heptapeptide being different from that of CCK-8, the effects of both forms on human pancreatic acinar Ca²⁺ signalling were found to be similar. The objective here, however, was to determine whether direct CCK responses occur in human pancreatic acinar cells.

In this study, normal human pancreatic tissue was transferred rapidly (<10 min) from within each patient to the laboratory, with protease inhibitors and pyruvate supplementation in the extracellular solution, and a brief period of collagenase digestion applied. Nonetheless, collagenase digestion is likely to be less effective in the large mammalian pancreas and to damage some acinar cells during isolation. The earlier work showed that tissue samples from obstructed, fibrotic pancreata were unsuitable for these experiments, as longer periods of collagenase digestion were required to gain isolated cells, which were morphologically abnormal with extensive trypsinogen activation spread throughout the cytosol, indicative of marked cellular injury from the isolation process. In isolated rat pancreatic acinar cells, prior pancreatic duct obstruction also diminished Ca²⁺ signalling (Mooren et al, 2003), perhaps due to receptor down-regulation. In a previous study of isolated human pancreatic acinar cells that did not

show functional CCK-elicited responses, transfer to the laboratory took longer and high concentrations of the cholinergic agonist carbachol (1 mM, equivalent to 100 μ M ACh) were required to elicit $[Ca^{2+}]_c$ changes (Ji et al, 2001). Also, the only $[Ca^{2+}]_c$ changes described in the previous study were sustained, not oscillatory (Ji et al, 2001). In contrast, the responses observed here are oscillatory Ca^{2+} signals in response to a low concentration of ACh (50 nM), of a similar order of magnitude to the Ca^{2+} signals elicited by CCK, at the very low concentrations typical of normal physiology (1-20 pM) (Walsh JH, 1987).

The characteristics of the Ca^{2+} signals, the coordinated increase in NADH autofluorescence and resulting exocytotic secretion evoked by CCK or ACh in human pancreatic acinar cells were similar to those in many previous studies of rodent cells (Petersen et al, 2008, 1992). For example, both CCK and ACh elicited oscillatory $[Ca^{2+}]_c$ elevations, always occurring locally in the apical pole and sometimes spreading globally as waves towards the base – again similar to that observed in rodent cells (Ashby et al, 2002). In human pancreatic acinar cells, therefore, some apical Ca^{2+} signals are likely to be contained by the peri-granular mitochondrial buffer barrier, as in rodents where these signals drive ATP generation (Petersen et al, 2008, Voronina et al, 2002, Rizutto et al, 2006, Petersen et al, 2006). Stronger Ca^{2+} signals may spread beyond this mitochondrial belt into the basolateral region, stimulating not only peri-granular but also peri-nuclear and sub-plasmalemmal mitochondrial ATP production. This pattern is entirely characteristic of the effects of CCK and ACh in murine pancreatic acinar cells, both isolated and within intact pancreatic preparations (Petersen et al, 2008). The $[Ca^{2+}]_c$ rise in the apical pole is critical for both enzyme and fluid secretion

from acinar cells, well established from studies on rodent cells to depend on the concentration of IP₃Rs in this part of the cell (Petersen et al, 2008, Nathanson et al, 1994). The importance of IP₃Rs for pancreatic secretion has been established previously in a study demonstrating that double knock-out of type 2 and 3 IP₃Rs in mice abolished pancreatic secretion in response to both ACh and CCK stimulation (Futatsugi et al, 2005). The data demonstrated here, shows that the IP₃R antagonist caffeine blocks CCK-elicited human pancreatic acinar cell Ca²⁺ signals which is consistent with the view that human cells work in a similar manner. A further similarity was the pattern of [Ca²⁺]_C changes and diminished secretion induced by CCK hyperstimulation. CCK hyperstimulation of isolated human pancreatic acinar cells featured a prolonged, global transient followed by an elevated plateau, a pattern associated with disordered secretion and implicated in the pathogenesis of pancreatitis (Kruger et al, 2000, Raraty et al, 2000, Saluja et al, 2007).

The demonstration of functional CCK receptors in human pancreatic acinar cells is a key finding underlining the relevance and significance of *in vitro* and *in vivo* rodent work that examines the role of CCK in pancreatic exocrine function and disease (Peterson et al, 2008, Saluja et al, 2007, Jensen et al, 2002, Pandol et al, 2003, Gukovsky et al, 2004, Husain et al 2005). This also confirms the potential of experiments on isolated acinar cells obtained from normal human pancreatic tissue to discover key mechanisms in cells presumed to be healthy as well as in those subject to injury in the form of clinical acute pancreatitis.

CHAPTER 4

**Toxic transformation of second messenger receptor
intracellular Ca²⁺ release in murine pancreatic acinar
cells**

Abstract

Background and Aims:

Sustained, toxic elevations of the acinar cytosolic free calcium ion concentration ($[Ca^{2+}]_c$) are implicated in acute pancreatitis. The intracellular mechanisms of this elevation are as yet unclear and further understanding of the mechanisms could determine potential therapeutic targets to halt disease progression.

Methods:

Isolated mouse pancreatic acinar cells were loaded with fluorescent dyes for confocal microscopy to measure $[Ca^{2+}]_c$ (Fluo 4), ADP:ATP ratio (Mg Green) and NADH autofluorescence in response to secretagogues and intracellular second messengers at normal physiological concentrations (CCK, ACh, IP_3 , NAADP, cADPR), together with low doses of palmitoleic acid ethyl ester (POAEE) and tauro lithocholic acid (TLCS) ($10\mu M$). In some experiments high dose TLCS ($200\mu M$) alone was used. Whole-cell patch clamp was used to measure the calcium-activated chloride current and apply or withhold ATP intracellularly. Cell viability at the conclusion of each experiment was assessed using propidium iodide staining (PI).

Results:

Toxic transformation of secretagogue induced physiological Ca^{2+} occurred with the perfusion of low doses of TLCS and POAEE resulting in cell injury. The intracellular second messengers implicated are IP_3 , cADPR and NAADP with the IP_3 receptor channel crucial with both toxins. However, if supplementary ATP is added to the intracellular milieu, cellular injury is avoided with continued

extrusion of large quantities of Ca^{2+} from the cytosol indicating functional Ca^{2+} ATPase pumps. This is not the case in cells which do not receive supplementary ATP.

The toxic sustained Ca^{2+} elevation can also be prevented by the removal of external Ca^{2+} or blockade of IP₃ receptors using caffeine, with cell injury again being avoided. Therefore it can be deduced that it is the large, sustained toxic $[\text{Ca}^{2+}]_c$ load which causes mitochondrial impairment and subsequent lack of ATP production leading to Ca^{2+} ATPase pump failure and cell death.

Conclusions:

Physiological concentrations of secretagogues, in combination with low dose toxins (TLCS/FFAE) increase $[\text{Ca}^{2+}]_c$ by changing the open state probability of Ca^{2+} release channels. The inositol trisphosphate receptor is common to both toxins and through Ca^{2+} ATPase pump failure from impaired mitochondrial ATP production, causes cell injury. The stimulus combinations used are more representative of what may occur clinically, and therefore lowering sustained intracellular $[\text{Ca}^{2+}]_c$ by blockade of IP₃ receptor channels may reduce cell injury in pancreatitis.

Introduction

Ca^{2+} is a ubiquitous intracellular messenger that initiates many different biological responses through voltage-gated, receptor-mediated or store-operated entry from the cell exterior and second messenger-mediated Ca^{2+} release from internal stores (Sutton et al, 2003). The control of rapid, transient fluxes of Ca^{2+} depends on the regulation of Ca^{2+} channels between sub-cellular compartments with large, energy-dependent differences in Ca^{2+} concentrations. These mechanisms are normally highly efficient, but Ca^{2+} channel regulation is vulnerable to effects of noxious stimuli directly and/or indirectly.

Typically, cytosolic Ca^{2+} overload that initiates cell death is sustained, and is accompanied by other stresses that may include high levels of reactive oxygen species (ROS) and/or non-esterified fatty acids (FA) (Raraty et al, 2000, Criddle et al, 2004, 2006). The cause of Ca^{2+} overload (e.g. hyperstimulation, ATP production failure), the magnitude of change in $[\text{Ca}^{2+}]_c$ and its extent throughout the cell and how long the change is sustained with other stresses are present results in either programmed (apoptotic) or unregulated (necrotic) cell death (Raraty et al, 2000, Baumgartner et al, 2007), with more extreme Ca^{2+} loads making necrosis more likely. Many non-excitabile cells terminate Ca^{2+} signals by cytosolic clearance with low affinity, high capacity, plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchangers and high affinity, low capacity, Ca^{2+} -ATPase pumps. Some non-excitabile cells with little significant plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchange activity such as those of the pancreas, depend on significantly smaller and more localised Ca^{2+} signals to permit Ca^{2+} clearance and avoidance of Ca^{2+} accumulation. As such, however, these cells may be at particular risk of Ca^{2+} overload,

should either excessive Ca^{2+} entry or Ca^{2+} -ATPase pump failure occur. G-protein-linked receptor-mediated generation of the second messengers inositol trisphosphate (IP_3), cyclic ADP ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) evokes release of Ca^{2+} through IP_3 receptors (IP_3Rs), ryanodine receptors (RyRs) and most likely NAADP receptors (NAADPR) respectively, although the identity of a specific NAADPR remains elusive (Cancela et al, 2000, 2002, Gallione et al, 2006). Their receptors are differentially distributed in the membranes of the endoplasmic reticulum (ER, predominantly IP_3Rs and RyRs) and other intracellular Ca^{2+} stores, including lysosomes (NAADPRs) and secretory granules (IP_3Rs and RyRs). Ca^{2+} -induced Ca^{2+} release (CICR) spreads the signals, notably via RyRs. Second messengers evoke predominantly small, transient, localised increases of $[\text{Ca}^{2+}]_c$, and less often more extensive Ca^{2+} signals that coordinate normal activities throughout the cell (Cancela et al, 2002, Ashby et al, 2000). Under appropriate circumstances such Ca^{2+} signals may induce programmed cell death. There is a risk, however, that certain compounds might alter the open probability of IP_3Rs or RyRs, especially during binding of their physiological ligands, such that the resulting uncontrolled Ca^{2+} release could damage cells by inducing collapse of mitochondrial membrane potential ($\Delta\psi_m$) and subsequent necrosis. Here the results demonstrated below show that toxic transformation of second messenger-generated Ca^{2+} release from intracellular stores occurs in pancreatic acinar cells that have negligible plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ exchange. Both G-protein-linked receptor-mediated generation of second messengers and direct patch pipette delivery of specific second messengers (IP_3 , cADPR, NAADP) evoked physiological Ca^{2+} signals that were transformed into sustained, generalised Ca^{2+} release by toxins known to

induce the human form of pancreatitis. Transformation resulted in cellular necrosis, prevented by exclusion of Ca^{2+} from the cell exterior, specific antagonism of second messenger-mediated Ca^{2+} release, or patch pipette delivery of supplementary ATP. Transformation by different toxins was specific to different second messengers, confirming the potential for a variety of deleterious effects from different toxins on second messenger receptor Ca^{2+} channels.

Materials and Methods

Cell Preparation and Solutions

Freshly isolated pancreatic acinar cells and acinar cell clusters of two or three cells were prepared from the pancreases of adult CD1 mice using collagenase (Worthington Biochemical Corporation, Lakewood, NJ) as described previously (Criddle et al, 2006). All experiments were performed at room temperature (23-25°C) and all cells were used within 4 hours of isolation. The extracellular solution contained (mM): 140 NaCl, 4.7 KCl, 1.13 MgCl₂, 1 CaCl₂, 10 D-glucose, 10 HEPES (adjusted to pH 7.35 using NaOH); in some experiments CaCl₂ (calcium-free extracellular solution) or CaCl₂ as well as MgCl₂, (calcium- and magnesium-free extracellular solution) were omitted (all chemicals highest grade available from Sigma, Gillingham, UK). Standard whole-cell patch-clamp current recordings (as described below) were performed using an internal pipette solution of (mM): 140 KCl; 1.5 MgCl₂; 2 MgATP; 10 HEPES; 0.1 EGTA, pH 7.2. In some experiments MgATP was omitted from the internal pipette solution, while in others this was raised to 4 mM.

Patch-clamp current recording

The whole-cell configuration of the patch-clamp technique was used to deliver second messengers (IP₃, cADPr or NAADP) into single pancreatic acinar cells and/or record Ca²⁺-activated Cl⁻ currents as described previously in chapter 2 (see pages 126-127)

Confocal imaging

Confocal imaging of cells loaded with fluorescent dyes was performed using a Zeiss LSM510 system (Carl Zeiss Jena GmbH, Germany) or Leica AOBS SP2 system (Leica Microsystems AG, Germany for Mg Green experiments to measure the intracellular free ionised magnesium concentration, $[Mg^{2+}]_c$, for determination of ATP changes) simultaneously with patch clamp recording to assess the effects of a bile salt (TLCS) or fatty acid ethyl ester (POAEE, dissolved in 10 mM ethanol) superimposed on secretagogue (acetylcholine, ACh; cholecystokinin-8, CCK) or second messenger stimulation. Cells were loaded with fluorescent dyes as cell-permeable, hydrophobic esters (-AM), which accumulate following hydrolysis, rendering them hydrophilic. Fluo 4-AM (2.5 μ M) was loaded for 30 min at room temperature to measure $[Ca^{2+}]_c$. The fluorescence of Fluo 4 was excited using an argon 488 nm laser line, with emitted light collected using an LP505 filter (515-540 nm). The images collected were composed of 256 x 256 pixels, and the optical section selected to be 5-6 μ m. A C-Apochromat x 63 objective with a numerical aperture of 1.2 was used in all experiments, and image analysis carried out using Zeiss confocal LSM510 or Leica confocal AOBS SP2 image software. Some experiments were performed using the multi-track configuration of the LSM510 system which allowed simultaneous measurement of $[Ca^{2+}]_c$ (excitation 488 nm; emission 515-540 nm) and NADH autofluorescence (excitation 351 nm; emission 385-470 nm) to obtain a measure of mitochondrial metabolism. Further experiments were done to determine changes of intracellular ATP concentrations after loading with Mg Green-AM (4 μ M) for 30 min at room temperature. Since ATP has a ten-fold greater affinity for Mg^{2+} than ADP and most intracellular magnesium is present as MgATP, this

dye monitors changes in ADP: ATP ratios that may indicate maintenance or depletion of intracellular ATP supplies, the latter demonstrated by an increase in Mg Green fluorescence (excitation 476 nm, emission 500–550 nm) as $[Mg^{2+}]_c$ rises in the presence of a Ca^{2+} - and Mg^{2+} -free extracellular solution. At the end of experiments assessing ATP changes with Mg Green-AM, the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10 μ M) was added to induce complete mitochondrial depolarisation and terminate ATP production.

Cell toxicity measurement

After examination of cytosolic Ca^{2+} changes, Cl^- currents and NADH responses under various conditions, cells were incubated in propidium iodide (1 μ M P.I.) for 10 min, and observed by confocal microscopy (Zeiss LSM510; excitation 363 nm, emission >400 nm) to detect necrotic cell death.

Results

Low dose toxins alone do not cause pathological insult on rodent pancreatic acinar cells

Previous studies have demonstrated that low dose bile salts (TLCS) and ethanol or non-oxidative ethanol metabolites (POAEE) do not induce Ca^{2+} release in the cytosol or result in cell damage (Criddle et al, 2004, Voronina et al, 2002). This experimental data, however, did not examine the effect of the cell being in direct contact with the patch pipette attached as part of the whole cell configuration and, more importantly, whether this had any adverse effect on the cell.

Several control experiments demonstrated this not to be the case (see figure 4.1A).

From the control experiments, we were able to deduce that the concentrations of TLCS to be used as part of the experimental procedure was $10\mu\text{M}$ as this did not induce any $[\text{Ca}^{2+}]_c$ signals when perfused externally alone. With the same assumptions, the concentration of POAEE to be used was also $10\mu\text{M}$ dissolved in 10-20mM ethanol ($\text{C}_2\text{H}_5\text{OH}$), as this concentration consistently did not induce any $[\text{Ca}^{2+}]_c$ signals when perfused externally around the acinar cell when attached to a patch pipette.

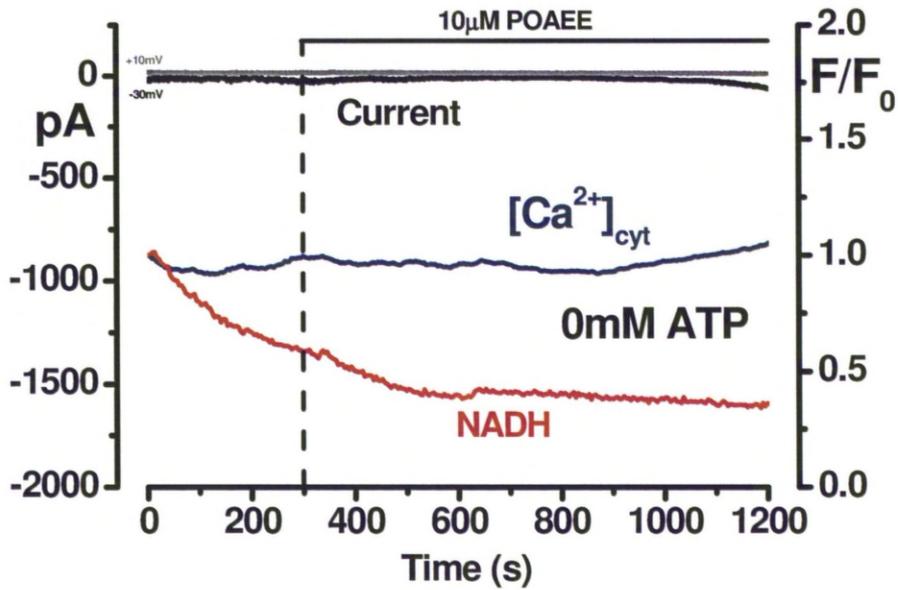
Prior to investigating the fatty acid ethyl esters, POAEE, it was important to establish a concentration of ethanol which does not result in a pathological Ca^{2+} signal generation with each of the second messengers as POAEE is soluble in ethanol and DMSO (Criddle et al, 2004, 2006). The resultant experimental data found no change in the Ca^{2+}

signal induced by the physiological concentrations of second messengers with ethanol concentrations of 10mM (n= 34/40) (figure 4.1B). Indeed in most situations we did not find an amplification of the Ca^{2+} signals with concentrations up to 50mM, but 10 -20mM was used to ensure no experimental bias.

A possible criticism of the electrophysiological technique used is whether ATP depletion occurs rapidly within the cell, when in the whole cell configuration, when no supplementary ATP (0mM ATP) is in the internal solution. Although experimental data presented later in this chapter provide suitable evidence against this criticism, the only way to prove categorically that this is not so is by measuring ATP direct or indirectly during patch clamp conditions. Unfortunately, there is no simple method by which ATP concentrations can be measured directly, but, by measuring magnesium concentration differences; one can indirectly infer what is happening to the intracellular ATP levels as ATP is bound to Mg^{2+} . As ATP is consumed, the free Mg^{2+} concentration in the cytosol increases.

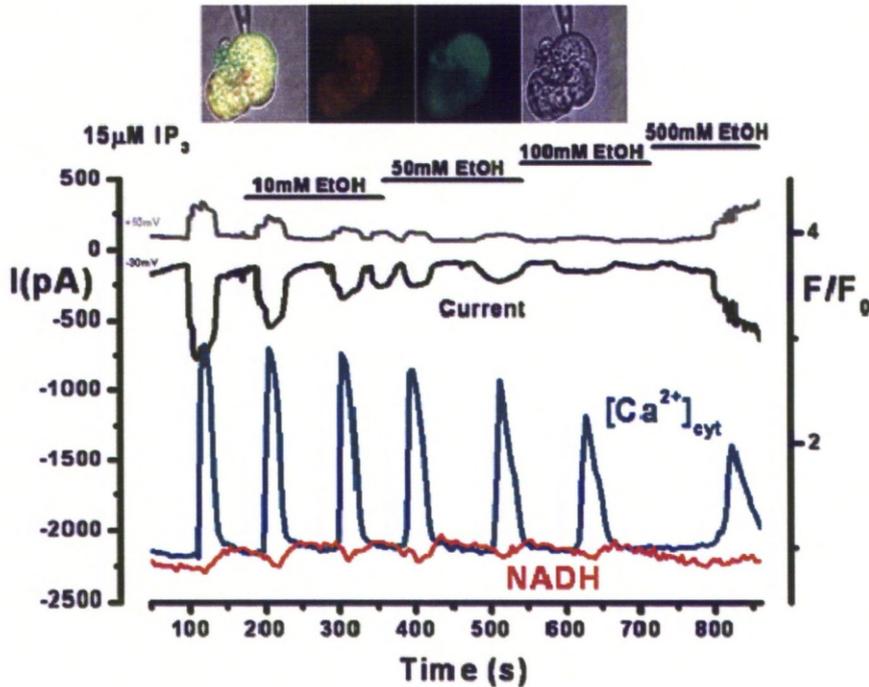
Several control experiments demonstrated below clearly show there is no rise in intracellular Mg^{2+} concentrations when the cell is connected to the patch pipette under whole cell conditions without physiological or pathological stimulation (figure 4.1C).

Figure 4.1 (A)



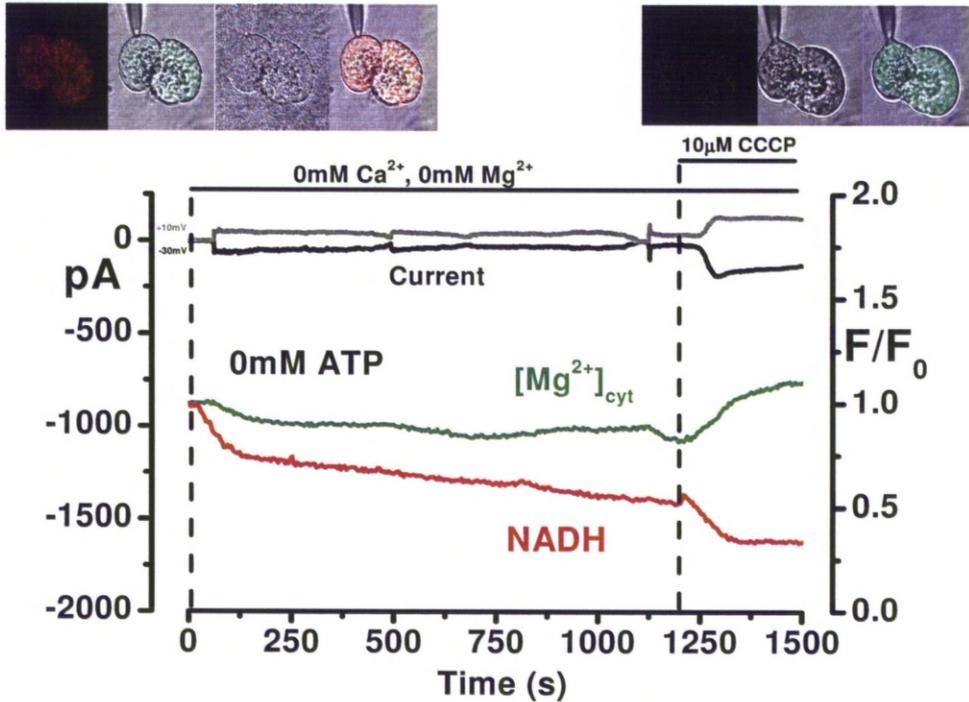
The concentration of the fatty acid ethyl esters ($10\mu\text{M}$) used did not trigger elevations of $[\text{Ca}^{2+}]_c$ (blue). Here the electrophysiological traces (black -30 mV ; grey $+10\text{ mV}$) are also unaffected by external perfusion with the fatty acid ethyl esters were dissolved in extracellular solutions containing $[10\text{mM} - 20\text{mM}]$ ethanol. Note normal NADH responses (red) are shown when the cells have a patch pipette attached containing internal solution.

Figure 4.1 (B)



The concentrations of ethanol used to dissolve the fatty acid ethyl esters did not alter physiological elevations of $[Ca^{2+}]_c$ induced by second messengers. Here elevations in $[Ca^{2+}]_c$ induced by $15\mu M$ IP_3 via the patch pipette are unaffected by increasing external perfusion with extracellular solution containing concentrations of ethanol from 10mM to 500mM. In subsequent experiments the fatty acid ethyl esters were dissolved in extracellular solutions containing [10mM - 20mM] ethanol. Note normal NADH responses are maintained to the large elevations of $[Ca^{2+}]_c$ in spite of increasing concentrations of ethanol. Inset shows experimental confocal images with the superior acinar cell attached to the patch pipette. The same image giving the information shows NADH (red), cytosolic Ca^{2+} (green) and normal transmitted light along the panel of images.

Figure 4.1 (C)



Control data showing a patched cell with 0mM ATP and no stimuli added. Note there is no appreciable rise in $[Mg^{2+}]_c$ until the mitochondrial membrane potential is collapsed with CCCP and the simultaneous collapse in NADH production. This demonstrates that the patch clamp process with 0mM ATP in the internal solution, itself, does not deplete the cell of its existing ATP stores.

The inset (left) represents the start of the experiment with: NADH autofluorescence, Mg^{2+} dye Mg^{2+} Green, transmitted light and overlay images. The inset (right) shows the end of the experiment with no NADH autofluorescence and an increase in free Mg^{2+} after collapsing the mitochondria membrane potential with the protonophore 10µM CCCP.

Secretagogue-elicited Ca^{2+} signals are transformed by low concentrations of Bile Salts (TLCS)

Ca^{2+} signals were recorded as changes in Fluo 4 fluorescence and Ca^{2+} -activated Cl^- currents that showed typically rapid, recurring elevations in $[\text{Ca}^{2+}]_c$ within the apical, granular pole of isolated pancreatic acinar cells in response to secretagogue stimulation.

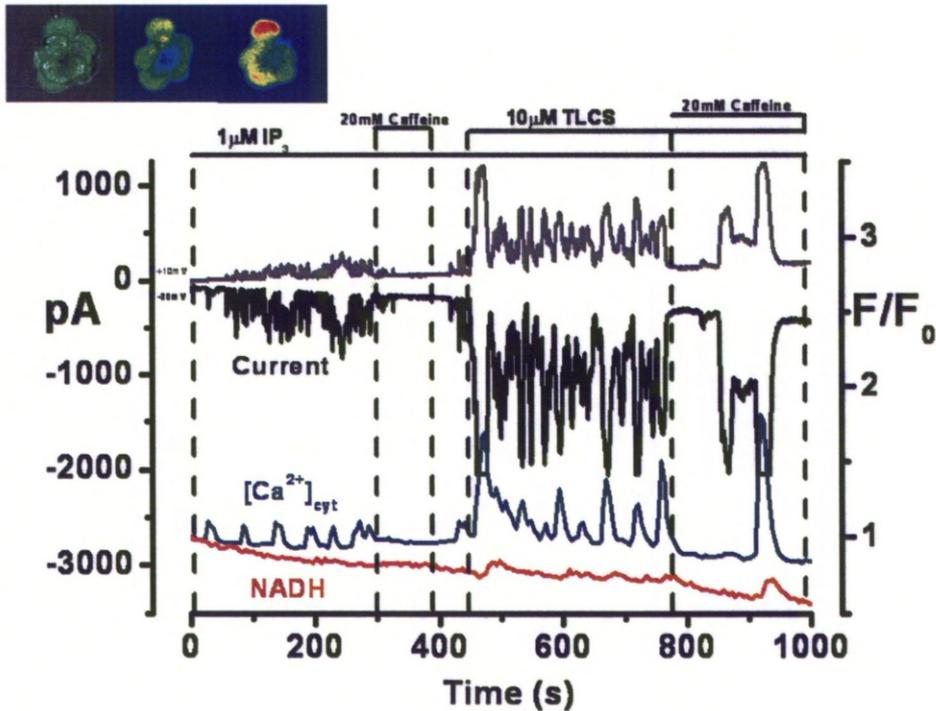
It is in the apical pole of the pancreatic cell that the signals trigger localised ATP production and secretion, spreading transiently to basolateral regions to coordinate ATP and protein synthesis throughout the rest of the cell. Such characteristic physiological signals are demonstrated (see figure 4.2A), by separate patch pipette delivery of the second messenger IP_3 and cADPR, or by cholecystokinin (CCK) binding to CCK1 receptors to elicit production of NAADP and cADPR, or by acetylcholine binding to muscarinic receptors (M3) eliciting intracellular IP_3 production as explained previously. Tauroolithocholate sulphate (TLCS) is one of several bile salts that induce necrosis through Ca^{2+} overload in unstimulated exocrine cells at concentrations $>100 \mu\text{M}$ (voronina et al, 2002).

This experimental data suggests that at such concentrations, bile salts induce abnormal IP_3R -mediated Ca^{2+} release, depolarise mitochondria and inhibit SERCA pumps, although the sequence and contribution of each mechanism to Ca^{2+} toxicity has not been resolved. At $10\mu\text{M}$, however, TLCS has minimal effects on $[\text{Ca}^{2+}]_c$ within unstimulated cells.

In clear contrast, Figure 4.2B shows that in cells stimulated by neurotransmitters (ACh) or hormones (CCK), or by patch pipette application of IP_3 , $10 \mu\text{M}$ TLCS caused

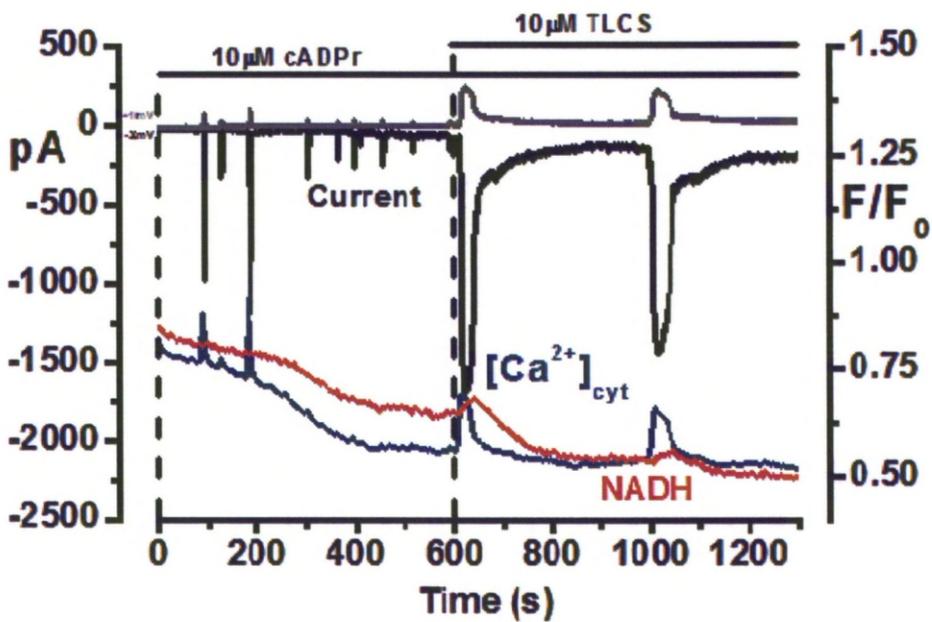
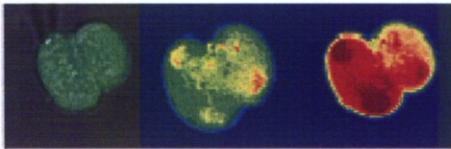
transformation of physiological Ca^{2+} signals into sustained (>30 s), global elevations of $[\text{Ca}^{2+}]_c$, that were cleared from the cytosol progressively less effectively. Transformation was found to be immediate and of large amplitude, spreading throughout the cell.

Figure 4.2 (A)



Normal physiological elevations of $[Ca^{2+}]_i$ (blue) induced by 1 μ M IP₃ via the patch pipette (black -30 mV; grey +10 mV) are blocked by the IP₃ receptor antagonist caffeine (20mM). Following cessation of perfusion with caffeine the normal elevations in $[Ca^{2+}]_c$ are transformed into global pathological elevations of $[Ca^{2+}]_c$ with the perfusion of a low concentration of TLCS. The global responses are only partially blocked by caffeine. Note NADH production and depletion in response to the large $[Ca^{2+}]_c$ rise. The inset shows a cluster of acinar cells with the patch pipette attached to the most superior supplying IP₃. The pseudo colour images show the cells before, where $[Ca^{2+}]_c$ elevations are evident in the patched cell, and during toxin stimulation with the patched cell showing a large rise in $[Ca^{2+}]_c$ and adjacent cells transiently increasing $[Ca^{2+}]_c$ in response to attached cell sustained $[Ca^{2+}]_c$ elevation.

Figure 4.2 (B)



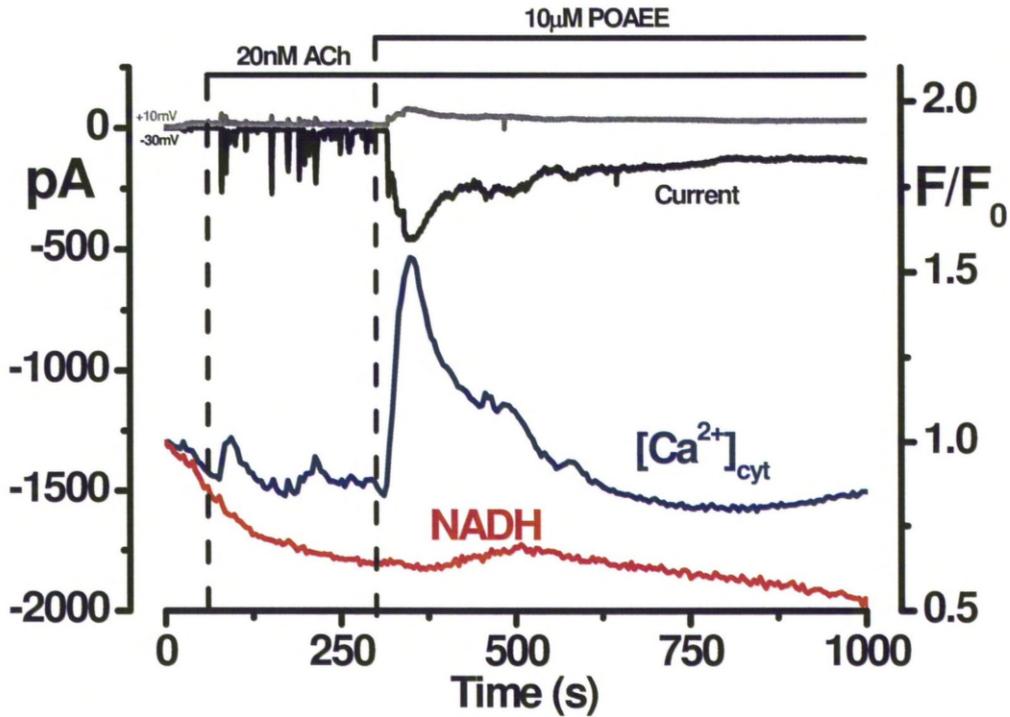
Normal physiological elevations of $[Ca^{2+}]_c$ induced by 10 μM cADPr via the patch pipette, are transformed into global pathological elevations of $[Ca^{2+}]_c$ with the perfusion of a low concentration of the bile salt TLCS. Note the NADH production and depletion in response to the large elevations of $[Ca^{2+}]_c$.

The pseudo colour images show the cells before toxic stimulation were $[Ca^{2+}]_c$ are evident in the patched cell, and during toxin stimulation with the patched cell showing a large rise in $[Ca^{2+}]_c$ and adjacent cells transiently increasing $[Ca^{2+}]_c$ in response to patched cell sustained $[Ca^{2+}]_c$ elevation.

Secretagogue-elicited Ca^{2+} signals are transformed by low concentrations of Fatty Acid Ethyl Esters (FAEE)

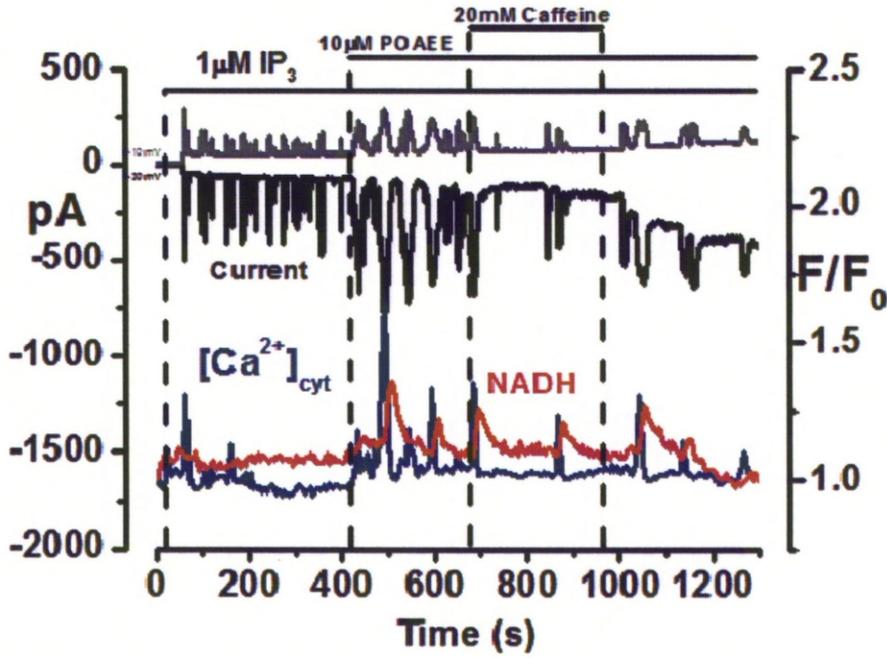
Fatty acid ethyl esters (FAEEs) are formed by non-oxidative synthase enzymes that catalyse the combination of FAs and ethanol, an alternative metabolic pathway which temporarily contributes to the clearance of ethanol in a number of organs, notably the pancreas and liver (Criddle et al, 2004). FAEEs, again at concentrations $>100 \mu\text{M}$, have been shown to induce necrosis in unstimulated pancreatic acinar cells through Ca^{2+} overload (Criddle et al, 2004, 2006). Although palmitoleic acid ethyl ester (POAEE) has been shown to open IP_3Rs , toxicity was found to be due primarily to FAEE hydrolysis and subsequent FA inhibition of mitochondrial ATP production. Nevertheless Figure 4.3 shows that $10\mu\text{M}$ POAEE transformed ACh-, CCK- and IP_3 -evoked physiological Ca^{2+} signals into prolonged, global elevations of $[\text{Ca}^{2+}]_c$. Transformation was again immediate, of large amplitude, spread throughout the cell and was sustained (>30 s), whichever of these stimuli was applied to evoke prior physiological Ca^{2+} signals. Also, transformed signals were progressively less well cleared from the cytosol, suggesting Ca^{2+} ATPase pump failure.

Figure 4.3 (A)



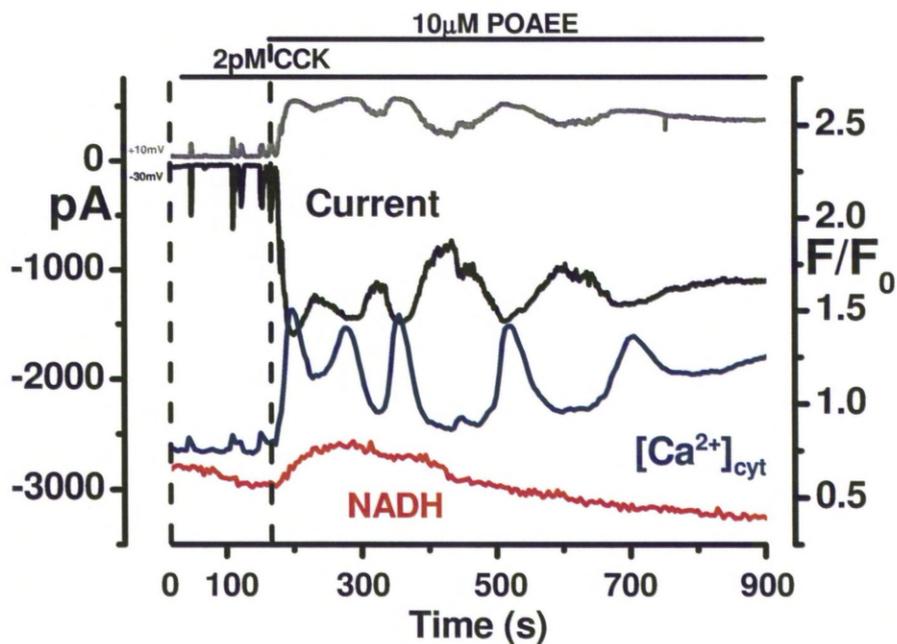
Normal physiological elevations of $[Ca^{2+}]_c$ (blue) induced by Acetylcholine at the physiological concentration (20nM). Normal intracellular physiological solution is present in the patch pipette containing 2mM ATP (black -30 mV; grey +10 mV). The physiological rises in $[Ca^{2+}]_c$ are transformed into a global sustained elevation with the perfusion of a low concentration of the POAEE. Note the NADH production and depletion in response to large elevations of $[Ca^{2+}]_c$.

Figure 4.3 (B)



Physiological elevations of $[Ca^{2+}]_c$ induced by $1\mu M$ IP_3 via the patch pipette are transformed into large global elevations of $[Ca^{2+}]_c$ with the perfusion of a low concentration of POAEE. The global responses are only partially blocked by 20 mM caffeine. From the previous data and data presented in this chapter with the second messengers, this indicates calcium release via the IP_3 receptor and NAADP/ryanodine receptor sensitization. Once perfusion with caffeine ceases, the larger global sustained responses resume. Note the NADH production and depletion in response to the large elevations of $[Ca^{2+}]_c$.

Figure 4.3 (C)



Normal physiological elevations of $[Ca^{2+}]_c$ induced by Cholecystokinin at the physiological concentration (2pM). The physiological rises in $[Ca^{2+}]_c$ are transformed into global sustained elevations with a low concentration of the POAEE. Note the NADH production and depletion in response to the large elevations of $[Ca^{2+}]_c$.

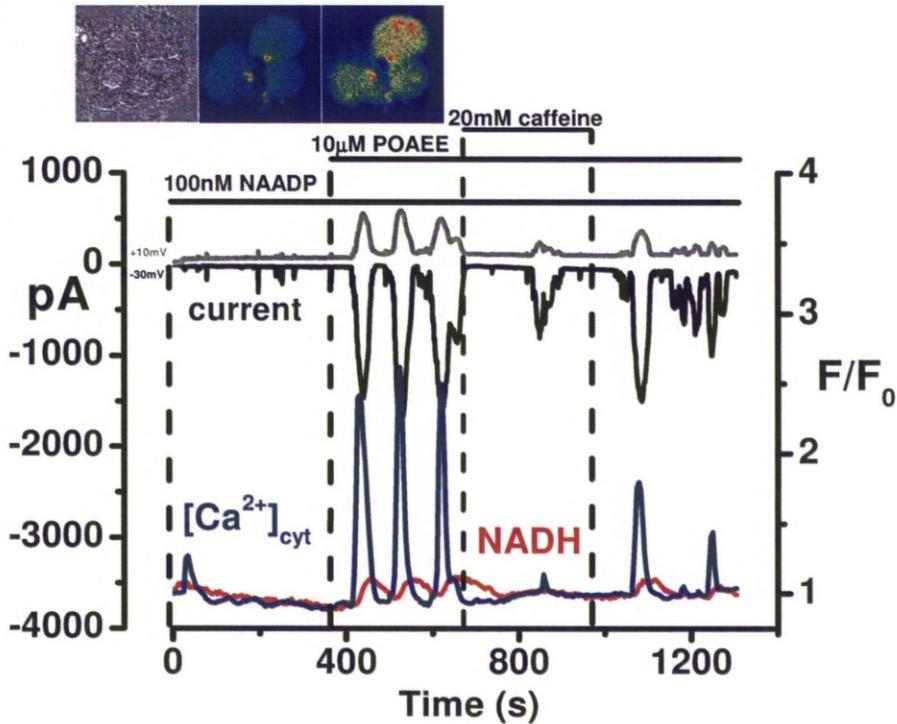
Second messenger receptor dependent calcium signal transformation patterns are specific to each toxin

Since the second messengers released by ACh differ from those released by CCK, (Cancela et al, 2000, 2002) I sought to determine the possible contribution of other second messenger Ca^{2+} channel release mechanisms to transformation. Numerous regulatory interactions occur that alter the open probability of second messenger receptor Ca^{2+} channels, notably between ligand binding sites and transmembrane domains. The comprehensiveness of these interactions may increase Ca^{2+} channel vulnerability to different toxins in more than one way. Figures 4.3 + 4.4 illustrates how different were found toxins to act differently to transform physiological second messenger-mediated Ca^{2+} release. Thus 10 μM TLCS transformed cADPR-evoked, but not NAADP-evoked Ca^{2+} signals, whereas 10 μM POAEE transformed NAADP-evoked, but not cADPR-evoked Ca^{2+} signals (Figure 4.4)

As observed with IP_3 -evoked Ca^{2+} signals, transformation was immediate, of large amplitude, global and sustained (>30 s). Recent data indicate that NAADP activates a separate receptor Ca^{2+} channel (NAADPR) that prompts CICR via IP_3 Rs and RyRs. Whether or not NAADP acts solely or predominantly on a separate receptor, the different effects of TLCS and POAEE on cADPR- and NAADP-evoked Ca^{2+} signals demonstrate that these toxins most likely act through different molecular interactions. The simplest hypothesis to account for our new data showing immediate, large amplitude, toxin-specific, global transformations of second messenger-evoked Ca^{2+}

signals, alongside our previous finding of second messenger Ca^{2+} channel release caused by high concentrations ($>100 \mu\text{M}$) of either toxin in unstimulated cells, is that the second messenger receptors are direct targets of these toxins.

Figure 4.4



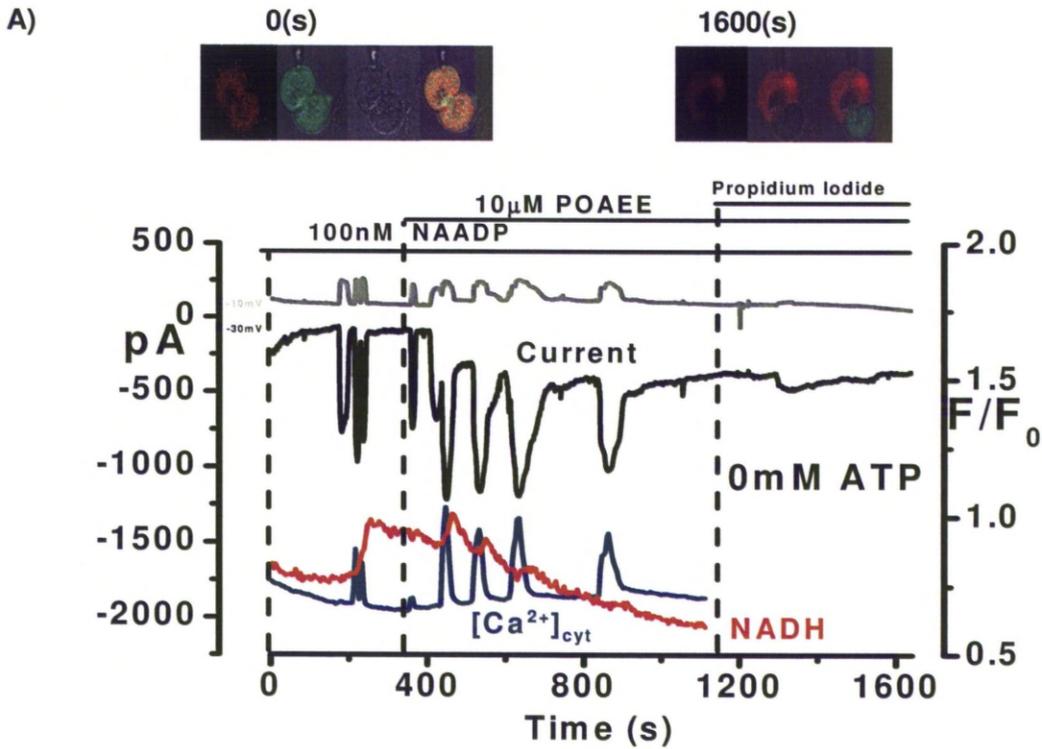
Physiological elevations of $[Ca^{2+}]_c$ induced by 100nM NAADP via the patch pipette are transformed into large global elevations of $[Ca^{2+}]_c$ with the perfusion of a low concentration of POAEE. The global responses are only partially blocked by 20 mM caffeine indicating calcium release via the IP₃ receptor and NAADP/ryanodine receptor sensitisation. Once perfusion with caffeine ceases, the larger global sustained responses resume. Note the NADH (red) production and depletion in response to the large elevations of $[Ca^{2+}]_c$. The pseudo colour images show the cells before, where $[Ca^{2+}]_c$ are evident in the patched cell, and during toxin stimulation showing a large rise in $[Ca^{2+}]_c$ and adjacent cells transiently increasing $[Ca^{2+}]_c$ in response to patched cell sustained $[Ca^{2+}]_c$ elevation.

Secretagogue-elicited global elevation of Ca^{2+} signals induce necrosis from cytosolic Ca^{2+} overload

To test whether toxic transformation of second messenger Ca^{2+} channel release induces necrotic cell death, NADH autofluorescence was assessed as a measure of ATP production and propidium iodide (PI) uptake as a measure of plasma membrane integrity within 30 min of the start of Ca^{2+} signalling. In all protocols tested in which a single secretagogue or second messenger was combined with a single toxin, transformation of physiological Ca^{2+} signals consistently resulted in reduced NADH autofluorescence, indicative of mitochondrial impairment, and PI uptake (see Table 4.1 and 4.2, Figure 4.5). Without Ca^{2+} -signal transformation the cell integrity and stimulus-metabolism coupling (NADH responses) were consistently preserved, while PI uptake was not observed. Thus cells were not detectably harmed by ACh (20 nM) or CCK (1-3 pM) in the concentrations applied, or pipette delivery of IP_3 (1-15 μM), cADPR (10 μM) or NAADP (100 nM), nor by TLCS (10 μM) or POAEE (10 μM) alone. In cells stimulated by pipette delivery of NAADP and exposed to 10 μM TLCS, no transformation occurred, NADH responses were maintained and no PI uptake resulted; similar findings were observed in cells stimulated by patch pipette delivery of cADPR and exposed to 10 μM POAEE. This data indicates that the transformation of the Ca^{2+} is toxic to the cell and induces necrosis (Figure 4.5). Cell necrosis from Ca^{2+} overload can also be prevented with the addition of supplementary ATP to the cell contents. Supplementary ATP (4 mM) applied via patch pipette protects cells from the toxicity of transformation, suggesting mitochondrial impairment is the principal consequence of $[\text{Ca}^{2+}]_c$ overload which ultimately determines cell fate (Figure 4.5).

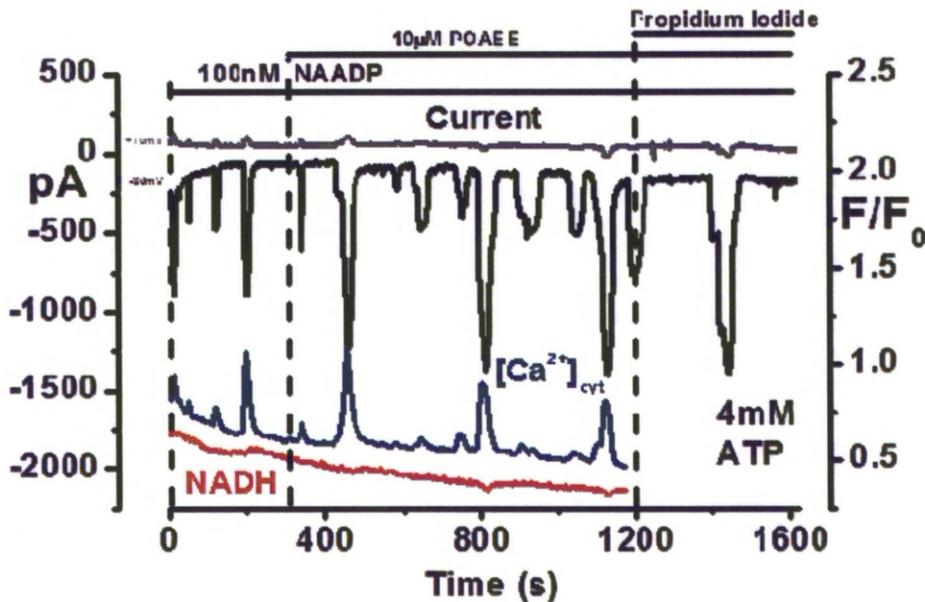
To exclude confounding effects from any trauma to cell membranes from the patch clamp technique, and from the entry of pipette solutions with or without ATP into the cell interior, intact isolated cells were exposed to physiologically representative concentrations of secretagogues (ACh or CCK) with or without either 10 μ M TLCS or 10 μ M POAEE, and PI uptake determined. Consistent with all results from cells with which a patch pipette is attached, addition of either toxin during secretagogue stimulation of intact cells resulted in a highly significant increase in PI uptake.

Figure 4.5



A) Quasi-physiological NAADP and 10 μ M POAEE induce global elevation of [Ca²⁺]_c followed by necrotic cellular changes detected by propidium iodide uptake when no extra ATP (0mM ATP) is added to the internal solution.

The left top panel represents images of: NADH autofluorescence, the calcium dye Fluo 4, transmitted light and overlay at the start (0 secs) of the experiment. In the right panel taken at the end of the experiment, note the cell attached to the patch pipette stains positive for propidium iodide (red stain) after POAEE application.



Using the same experimental set up, cell necrosis is avoided when supplementary ATP is supplied to the cell internally via the patch pipette. In this situation, global Ca^{2+} release occurs as before, but it is noticeable that there is not a rapid depletion of ATP indicated by a relatively stable NADH autofluorescence level which is able to respond to continuing global Ca^{2+} release. This means that the Ca^{2+} ATP dependent pumps located around the cell, but more specifically the SERCA pumps on the ER, and the plasma membrane Ca^{2+} pumps (PMCA'S), are able to extrude large loads of cytosolic Ca^{2+} preventing a cytotoxic overload triggering the cell death pathways (demonstrated by a lack of propidium iodide uptake). The results of the different stimulus and toxin combinations are demonstrated in table 4.1 and 4.2.

Table 4.1

Stimulus Combination	Potentialiation of $[Ca^{2+}]_i$	No potentialiation $[Ca^{2+}]_i$	0mM ATP	4mM ATP	Healthy	Necrotic
IP₃ (1-10μM) + TLCS (10μM)	31/33 cells	2/33 cells	N/A	N/A	N/A	N/A
IP₃ (1-10μM) + POAEE (10μM)	34/37 cells	3/37 cells	N/A	N/A	N/A	N/A
cADPr (10-15μM) + TLCS (10μM)	29/31 cells	2/31 cells	N/A	N/A	N/A	N/A
cADPr (10-15μM) + POAEE (10μM)	0/12 cells	12/12 cells	N/A	N/A	N/A	N/A
NAADP(50-100nM) + TLCS (10μM)	1/13 cells	12/13 cells	N/A	N/A	N/A	N/A
NAADP(50-100nM) + POAEE (10μM)	31/32 cells	1/32 cells	N/A	N/A	N/A	N/A
IP₃ /TLCS	-	-	6 cells	-	1/6 cells	5/6 cells
IP₃ /TLCS	-	-	-	4 cells	4/4 cells	0/4 cells
IP₃ /POAEE	-	-	9 cells	-	1/9 cells	8/9 cells
IP₃ /POAEE	-	-	-	4 cells	4/4 cells	0/4 cells
CADPr / TLCS	-	-	5 cells	-	0/5 cells	5/5 cells
cADPr / TLCS	-	-	-	4 cells	4/4 cells	0/4 cells
NAADP / POAEE	-	-	9 cells	-	1/9 cells	8/9 cells
NAADP / POAEE	-	-	-	5 cells	5/5 cells	0/5 cells

The second messenger / toxin combination producing pathological globalisation of Ca^{2+} signalling pathways. The cells stimulated with the toxic combinations were tested for cell viability with or without supplementary ATP in the pipette.

Table 4.2

Stimulus Combination	Potentialiation of $[Ca^{2+}]_i$	No potentiation $[Ca^{2+}]_i$	0mM ATP	4mM ATP	Healthy	Necrotic
ACh (20nM)/ TLCS	6/6 cells	0/6 cells	6 cells	-	0/6 cells	6/6 cells
ACh (20nM)/ TLCS	6/6 cells	0/6 cells	-	6 cells	6/6 cells	0/6 cells
ACh (20nM)/ POAEE	6/6 cells	0/6 cells	6 cells	-	0/6 cells	6/6 cells
ACh (20nM)/ POAEE	6/6 cells	0/6 cells	-	6 cells	6/6 cells	0/6 cells
CCK (1-5pM)/ TLCS	5/5 cells	0/5 cells	5 cells	-	0/5 cells	5/5 cells
CCK (1-5pM)/ TLCS	5/5 cells	0/5 cells	-	5 cells	5/5 cells	0/5 cells
CCK (1-5pM)/ POAEE	7/7 cells	0/7 cells	7 cells	-	0/7cells	7/7 cells
CCK (1-5pM)/ POAEE	5/5 cells	0/5 cells	-	5 cells	5/5 cells	0/5 cells

The secretagogues / toxin combination which result in toxic globalisation of calcium signalling pathways. The potentiating combinations were tested for cell viability with or without supplementary ATP in the pipette.

Secretagogue – induced toxic Ca^{2+} signals causes mitochondrial impairment and subsequent cellular necrosis

Extracellular exogenous ATP was either added or subtracted from the patch pipette to further examine the dependence on intracellular ATP reserves which may influence whether the toxic Ca^{2+} transformation results in cellular necrosis.

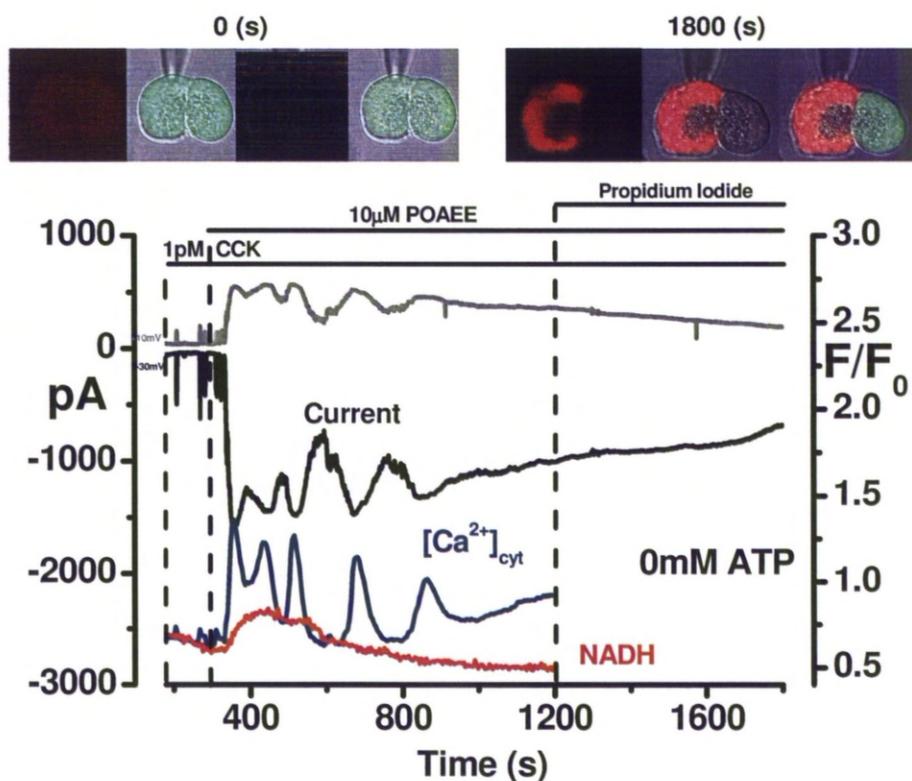
When external (0mM) ATP was omitted from the patch pipette contents, the Ca^{2+} signal transformation was large, immediate and quickly sustained as the Ca^{2+} ATPase (SERCA/PMCA's) were unable to extrude the large build up of $[\text{Ca}^{2+}]_c$. Cellular necrosis was consistently observed as demonstrated in Figure 4.6A. However, when supplementary ATP (4mM) was added the intracellular contents, the Ca^{2+} signal transformation was large but not sustained as the mitochondria were energised (as demonstrated by increased NADH autofluorescence) and able to produce ATP as well as the supplementary ATP being available for the Ca^{2+} ATPase pumps to extrude the large $[\text{Ca}^{2+}]_c$ into the ER and outside the cell with stimulus-metabolism coupling maintained. In this situation no cellular toxicity was demonstrated (Figure 4.6B).

From this experimental data set, a possible explanation is that it is the mitochondrial impairment and consequent impaired ATP production that is the pivotal pathological process. It has been previously postulated that the Ca^{2+} causes direct Ca^{2+} ATPase pump failure directly (Lee et al, 2002), but should this be the case the addition of supplementary ATP would not have any influence of the pumps ability to function, which is clearly not the case as demonstrated in the experiments below (Figure 4.6).

This important point needed to be further clarified, which was performed by perfusing the cells with a high concentration of TLCS (200 μM) – shown to be deleterious to the

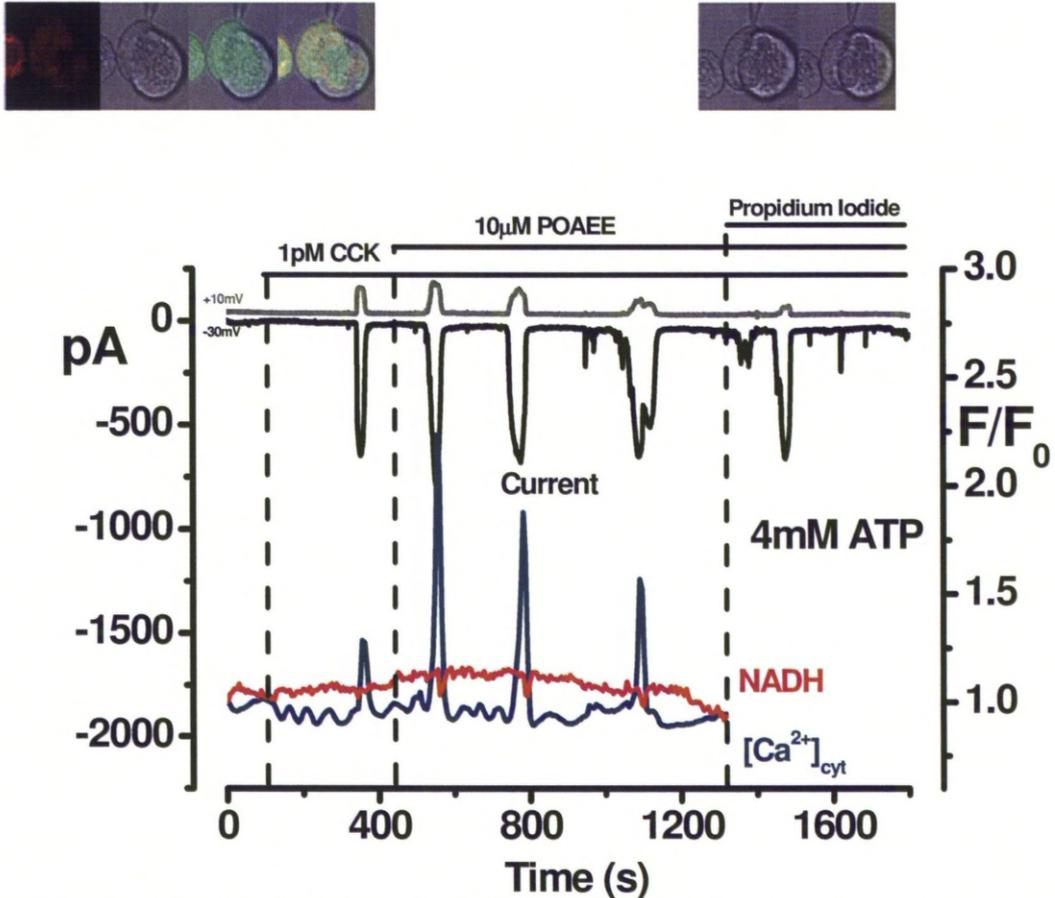
pancreatic acinar cell. Figure 4.6C demonstrates a non-patched cell undergoing a toxic transformation in response to the noxious secretagogue stimulation resulting in a sustained elevation in $[Ca^{2+}]_o$ and subsequent cell necrosis. But, in a cell in close proximity to that undergoing necrosis, that cell has supplementary ATP (4mM) added via the patch pipette and is able to extrude large concentrations of $[Ca^{2+}]_o$ via functional Ca^{2+} ATPase pumps and no cell injury occurs providing good evidence that it is mitochondrial impairment which leads to Ca^{2+} ATPase pump failure. Indirect measurements of ATP production and consumption are demonstrated later in this chapter.

Figure 4.6 (A)



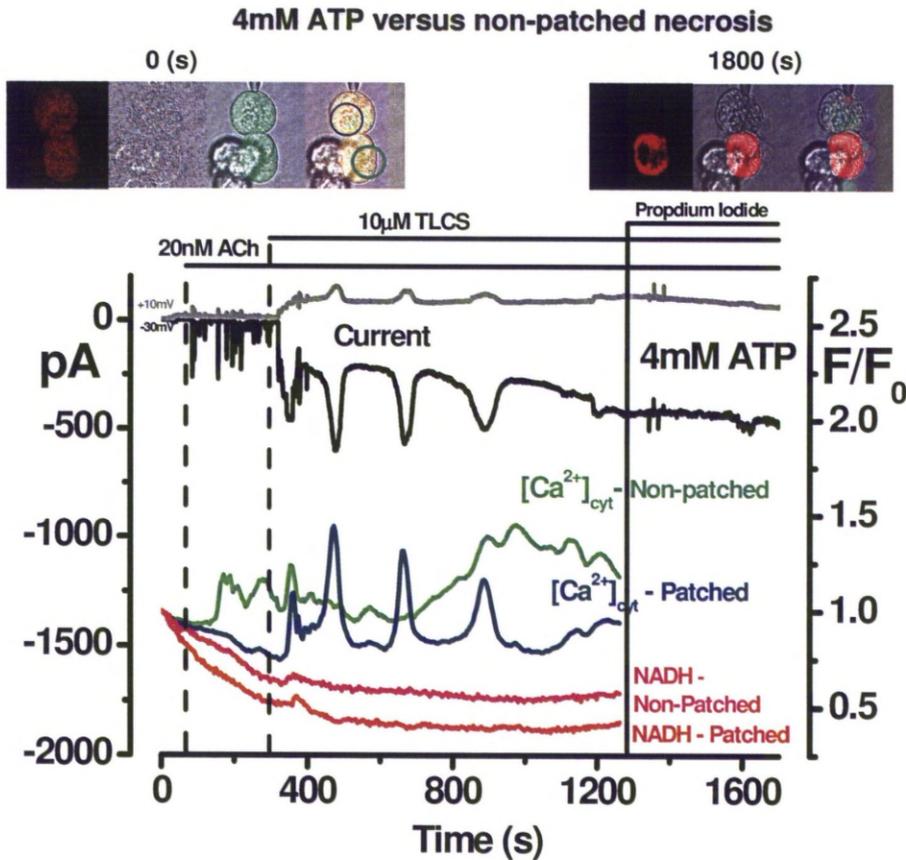
Quasi-physiological CCK (1pM) and 10μM POAEE induce global $[Ca^{2+}]_c$ release followed by necrotic cellular changes detected by propidium iodide uptake when no extra ATP (0mM ATP) is present. Note the decline of NADH autofluorescence that is unable to respond to the global cytosolic Ca^{2+} release, indicating no ATP production and subsequent sustained pathological rise in intracellular Ca^{2+} . The left top panel represents: NADH autofluorescence, Ca^{2+} dye Fluo-4, transmitted light and overlay at the start. The right panel taken at the end: note only the cell attached to the patch pipette containing 0mM ATP stains positive for propidium iodide (red) after POAEE application.

Figure 4 (B)



Quasi-physiological CCK and 10 μ M POAEE induce global elevations of [Ca²⁺]_c but when 4mM ATP was added to the internal solution a sustained [Ca²⁺]_c elevation did not occur. There is no significant drop in NADH and there is consumption followed by production of ATP in response to the global [Ca²⁺]_c. Note there is no sustained elevation in [Ca²⁺]_c as measured by both fluorescence and electrophysiologically as the ATP dependent Ca²⁺ pumps are able to keep working in the presence of extra ATP. This is confirmed by no necrotic cellular change detected with a lack of propidium iodide uptake.

Figure 4.6 (C)



In most experiments a significant number of cells in the field of view were also necrotic at the end of the experiment. Therefore, when quasi-physiological ACh (20nM) and 10µM TLCS induced global $[Ca^{2+}]_c$ elevations in both the patched (blue line) and non-patched cell (green line), it is the cell with the extra ATP supplied which prevents a sustained rise in $[Ca^{2+}]_c$ and prevent necrotic cellular changes as detected by PI uptake (right panel). NADH autofluorescence in the patch cell (red line) is still able to respond to global Ca^{2+} release indicating ATP production, but not in the adjacent cell (pink line).

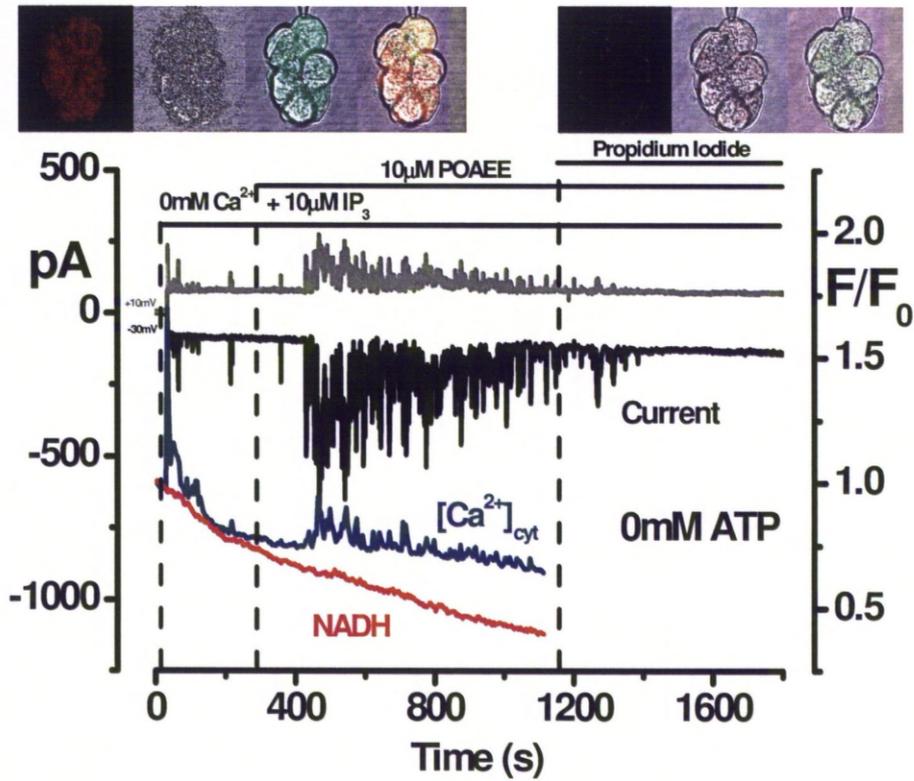
Toxicity of transformed secretagogue-elicited Ca^{2+} signals is also dependent on cellular Ca^{2+} entry as well as mitochondrial impairment

Certain other possible factors which may influence the toxicity of Ca^{2+} signal transformation were also examined. The effects of extracellular Ca^{2+} removal, second messenger receptor blockade as well as strategies to prevent consequences of mitochondrial injury were addressed. When extracellular Ca^{2+} was omitted from the perfusate, Ca^{2+} signal transformation was immediate but of smaller magnitude and markedly less sustained, with oscillatory signals that declined as stores emptied (see Figure 4.7) No mitochondrial and cellular injury occurred as demonstrated by a lack of PI uptake. This is further demonstrated using Mg^{2+} green as an indicator for free Mg^{2+} concentration (indirectly measuring ATP consumption and production by measuring ATP:ADP ratio). NADH and Mg^{2+} green responses are short, robust and return to baseline in figure 4.7C indicating functional mitochondrial ATP production in response to $[\text{Ca}^{2+}]_c$ elevations in the absence of external Ca^{2+} . It is only when the protonophore CCCP is added with collapse of the mitochondrial membrane potential ($\Delta\psi_m$) do we observe a sustained rise of Mg^{2+} green with correspondent decline of NADH fluorescence.

Caffeine is an IP_3R antagonist, independent of its enhancement of CICR via the RyR, and application of caffeine specifically prevents IP_3 -evoked, but not cADPR-evoked, Ca^{2+} signals (Figure 4.8). During combined administration of IP_3 and TLCS, caffeine was found to inhibit almost all Ca^{2+} signalling; during IP_3 and POAEE administration, caffeine was similarly effective, although as seen in Figure 4.4, inhibition tended to

lessen after several minutes. Global, sustained Ca^{2+} signals were never seen during application of caffeine with these combinations, during which time NADH generation was sustained and subsequent PI uptake was not observed. In keeping with the known dependency of NAADP-evoked Ca^{2+} signals on CICR from IP_3Rs , caffeine also inhibited transformation of NAADP-evoked Ca^{2+} signals by POAEE (Figure 4.8). Caffeine did not, however, inhibit TLCS-induced transformation of cADPR-evoked Ca^{2+} signals. The effects of 0mM Ca^{2+} and of external caffeine perfusion are recorded in Table 4.3.

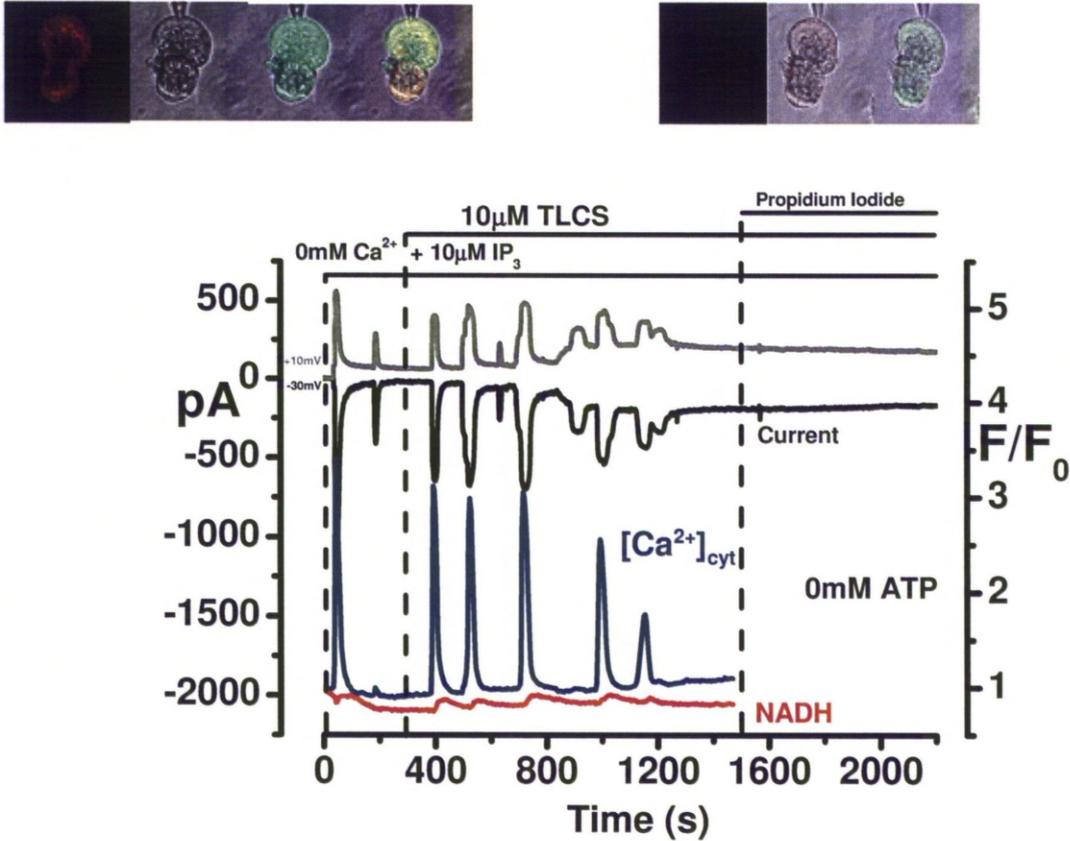
Figure 4.7 (A)



Normal physiological oscillations in $[Ca^{2+}]_c$ generated by IP₃ in the absence of external calcium are potentiated into faster, larger $[Ca^{2+}]_c$ transients with POAEE when ATP is absent, but not global sustained $[Ca^{2+}]_c$ elevations.

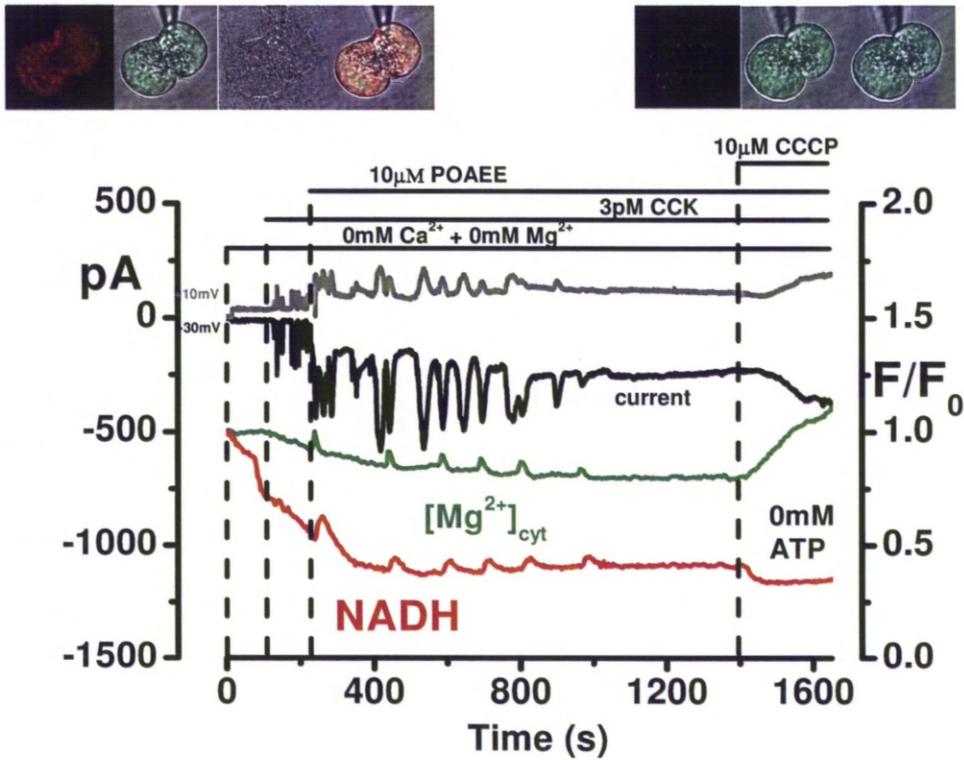
Cellular necrosis is avoided highlighting the critical role of calcium entry into the cell when necrosis is evident.

Figure 4.7 (B)



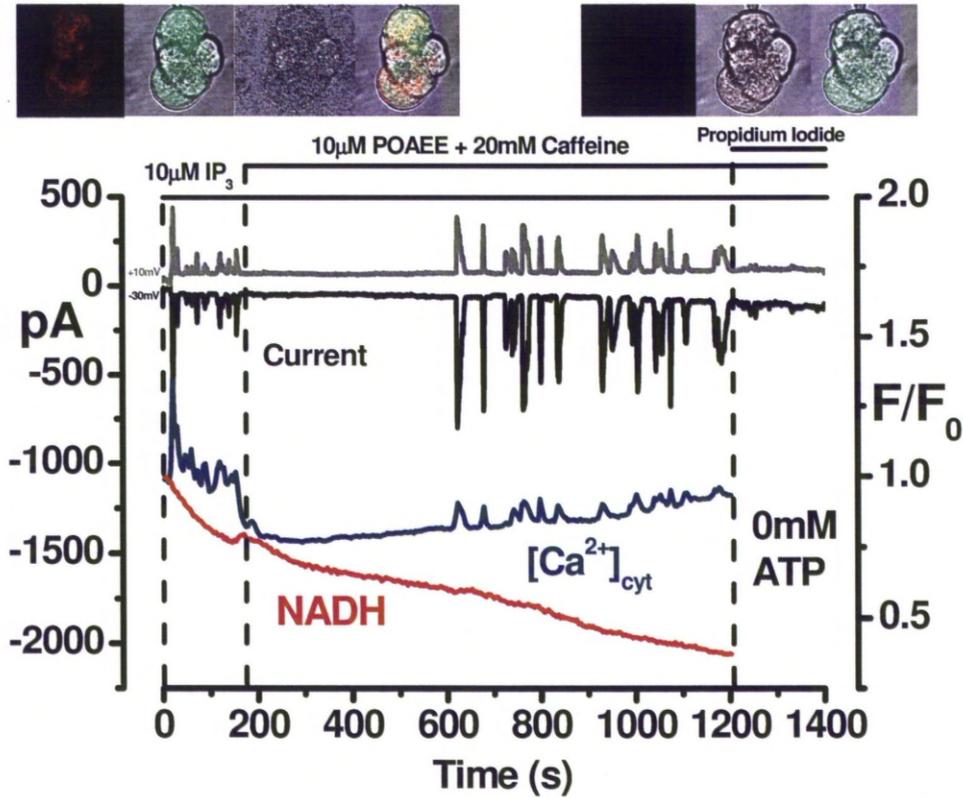
Normal physiological oscillations in $[Ca^{2+}]_c$ generated by IP_3 in the absence of external calcium are potentiated into slightly faster and larger $[Ca^{2+}]_c$ transients with TLCS when ATP is absent, but not global sustained $[Ca^{2+}]_c$ elevations. In the absence of sustained global elevations of cytosolic Ca^{2+} , the NADH autofluorescence increases in response to the Ca^{2+} signals indicating functional mitochondria, providing ATP to pump excessive Ca^{2+} out of the cytosol as reflected in the electrophysiological and fluorescence traces. Cellular necrosis is avoided also highlighting the critical role of calcium entry into the cell when necrosis is evident.

Figure 4.7 (C)



Transient rises in $[Ca^{2+}]_c$ are potentiated into global responses, not sustained in the presence of 0mM ATP in 0mM Ca^{2+} external solution. The free magnesium concentration ($[Mg^{2+}]_c$) (green) shows increases to each global response indicating consumption of ATP, but no sustained rise in $[Mg^{2+}]_c$ indicating ATP is still available, confirmed by the NADH. The NADH (red) responds and increases to the large $[Ca^{2+}]_c$. With the protonophore CCCP, there is collapse in NADH production and a rise in free $[Mg^{2+}]_c$ indicating functional mitochondria prior to CCCP, highlighting the importance of external Ca^{2+} with sustained calcium signal generation with ATP depletion.

Figure 4.8



Normal physiological oscillations in $[Ca^{2+}]_c$ are initially blocked when the IP_3 receptor antagonist caffeine (20mM) is perfused simultaneously with POAEE when ATP is absent in the internal solution. Following a period without any $[Ca^{2+}]_c$ elevations, normal signalling resumes in the presence of caffeine indicating $[Ca^{2+}]_c$ oscillations due to release via other receptors, most probably ryanodine/ NAADP receptors which are shown to be sensitised earlier. The normal signals have different kinetics than those provoked by IP_3 . The absence of global sustained $[Ca^{2+}]_c$ elevations protects against cellular necrosis in the absence of supplementary ATP confirming the importance of the IP_3 receptor in the very earliest events leading to pathological $[Ca^{2+}]_c$ release and subsequent necrosis.

Table 4.3

Stimulus/Toxin 0mM ATP	Simultaneous Caffeine (20Mm)	Subsequent Caffeine (20mM)	0mM external Ca ²⁺	Necrosis	No necrosis
1-10µM IP ₃ + 10µM POAEE	-	-	-	8/9 Cells	1/9 Cells
1-10µM IP ₃ + 10µM TLCS	-	-	-	5/6 Cells	1/6 Cells
1-10µM IP ₃ + 10µM POAEE	-	-	7 Cells	0/7 Cells	7/7 Cells
1-10µM IP ₃ + 10µM TLCS	-	-	7 Cells	1/7 Cells	6/7 Cells
1-10µM IP ₃ + 10µM POAEE	6 Cells	-	-	0/6 Cells	6/6 Cells
1-10µM IP ₃ + 10µM TLCS	5 Cells	-	-	1/6 Cells	5/6 Cells
1-10µM IP ₃ + 10µM POAEE	-	5 Cells	-	0/5 Cells	5/5 Cells
1-10µM IP ₃ + 10µM TLCS	-	6 Cells	-	1/6 Cells	5/6 Cells

The second messenger (IP₃) and toxin combination resulting in pathological globalisation of physiological calcium signals when supplementary ATP is absent. The potentiating combinations were tested for cell viability with or without either: external (zero) Ca²⁺ perfused, with the simultaneous perfusion of the IP₃ receptor antagonist caffeine, or caffeine perfused after the perfusion with the toxin. The results indicate that both caffeine and 0mM external Ca²⁺ is protective to the cell when ATP is absent.

Mitochondrial impairment is the principal consequence of intracellular toxic Ca^{2+} transformation

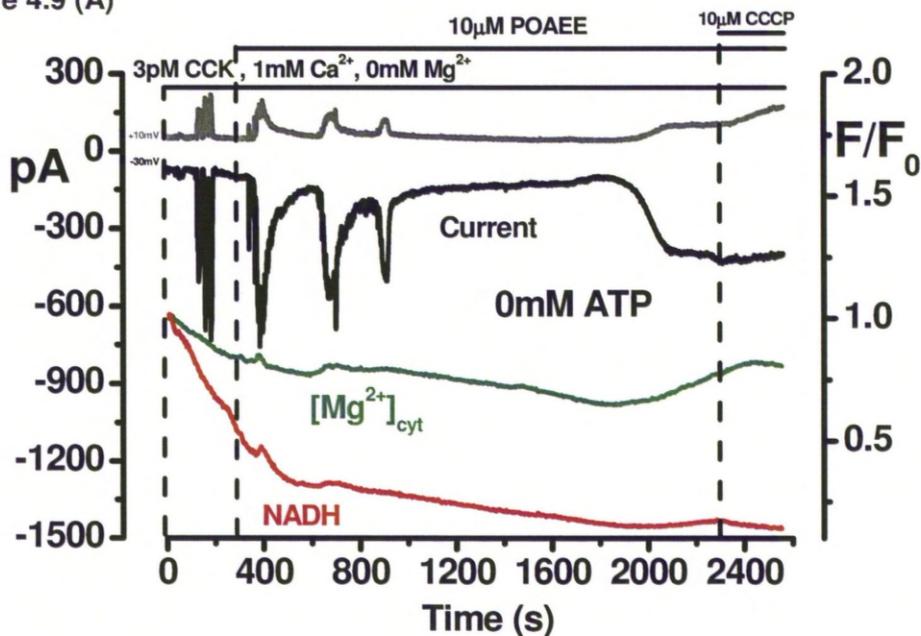
The experimental data demonstrate supplementary ATP (4 mM) protected cells from the toxicity of Ca^{2+} transformation, suggesting mitochondrial impairment to be the principal consequence of $[\text{Ca}^{2+}]_c$ overload determining cell fate (Figure 4.6). The importance of ATP in preventing necrosis was confirmed in all secretagogue (ACh or CCK) or second messenger (IP_3 , cADPR or NAADP) combinations with either toxin (Table 4.2 and 4.3). To explore possible changes in the intracellular ATP concentration ($[\text{ATP}]_c$) in patched cells under various conditions, experiments were undertaken with Mg Green, which detects changes in the ADP:ATP ratio. Without toxic transformation (and no supplementary ATP), $[\text{ATP}]_c$ was not measurably affected, whatever secretagogue /second messenger/toxin combination was applied.

Toxic transformation, however, consistently resulted in a significant increase in the ADP:ATP ratio that was not altered by subsequent application of the protonophore CCCP, which causes prompt collapse of $\Delta\psi_m$ (Figure 4.9A).

These results indicate that ATP supplementation prevents the toxicity of Ca^{2+} signal transformation primarily by compensating for ATP production failure, rather than by overcoming any direct, toxin-mediated inhibition of SERCA and/or PMCA pumps.

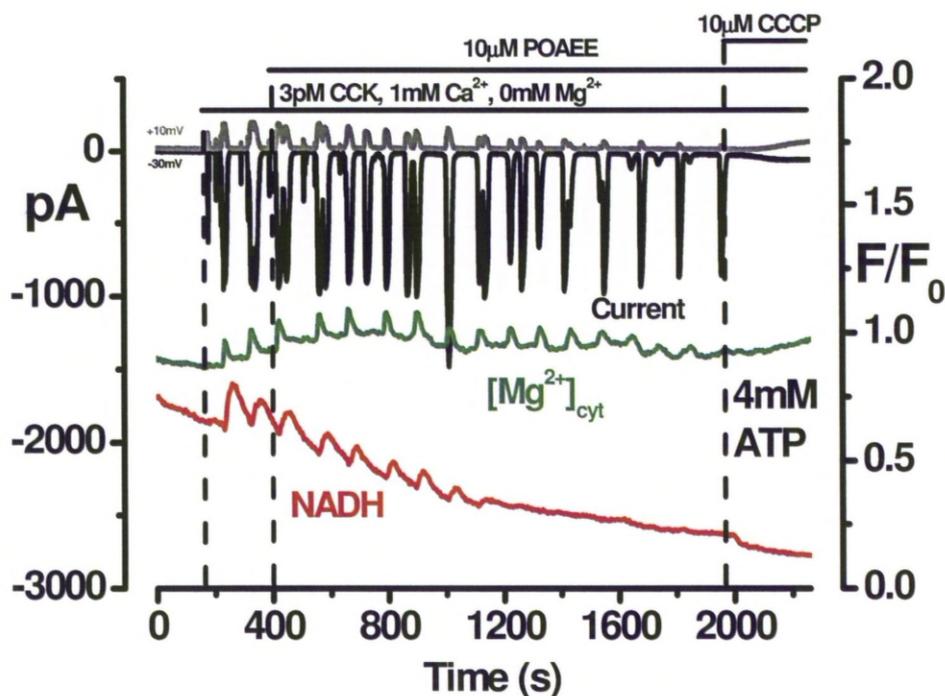
These data also confirm mitochondria to be the principal site of ATP production failure, since CCCP applied after toxic transformation did not alter Mg Green fluorescence (Figure 4.9A ,4.9B), (Table 4.4).

Figure 4.9 (A)



Transient rises in $[Ca^{2+}]_c$ are potentiated into global sustained responses as measured electrophysiologically. The free magnesium concentration ($[Mg^{2+}]_c$) (green) shows increases to each global response indicating consumption of ATP. Initially the NADH (red) responds and increases to the large $[Ca^{2+}]_c$ elevations but this is not long lasting. Eventually there is a large increase in $[Mg^{2+}]_c$ massive consumption of ATP with little or not production and is unaffected when the mitochondrial membrane potential is collapsed with the protonophore CCCP (10µM). This confirms the combination of global sustained increases in $[Ca^{2+}]_c$ together with ATP depletion and no production lead to cellular injury.

Figure 4.9 (B)



Transient rises in $[Ca^{2+}]_c$ are transformed into global which are not sustained in the presence of 4mM ATP. The free $([Mg^{2+}]_c)$ shows increases to each global response indicating consumption of ATP, but there is no sustained rise in $[Mg^{2+}]_c$ indicating ATP is still available which is confirmed by the NADH trace. Initially the NADH (red) responds and increases to the large $[Ca^{2+}]_c$ elevations but this is not long lasting and the additional ATP both energizes the mitochondria and supplies the ATP dependent Ca^{2+} pumps allowing large global elevations of $[Ca^{2+}]_c$ without a sustained rise. With CCCP, there is collapse in NADH production and a rise in free $[Mg^{2+}]_c$ indicating functional mitochondria prior to CCCP.

Table 4.4

Toxin/Stimulus Combination	Global sustained $[Mg^{2+}]_i$ increase prior to 10μM CCCP	0mM ATP	4mM ATP	Sustained $[Mg^{2+}]_i$ rise following 10μM CCCP at end of experiment	Necrosis
Control	0/9 cells	9 cells	-	3/3 cells	0/6 cells
1pM CCK / 10 μ M POAEE	6/6 cells	6 cells	-	0/6 cells	-
1pM CCK / 10 μ M POAEE	0/6 cells	-	6 cells	6/6 cells	-
1pM CCK / 10 μ M POAEE/ 0mM $[Ca^{2+}]_{ext}$	0/5 cells	5 cells	-	5/5 cells	-

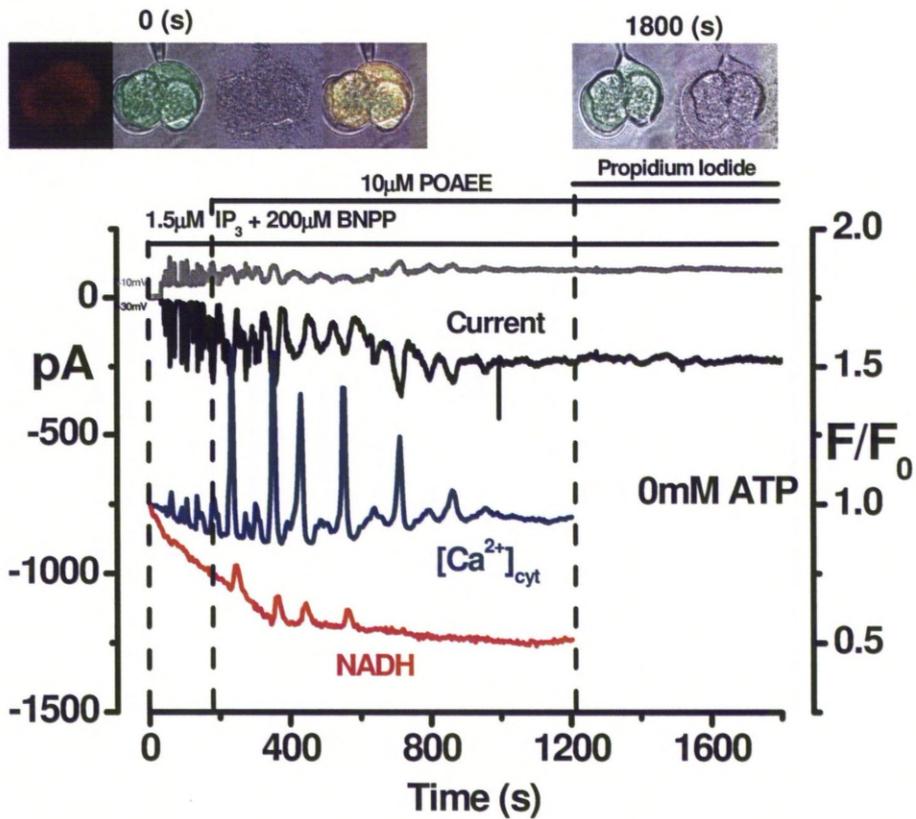
The secretagogues/ toxin combination resulting in a sustained rise in free $[Mg^{2+}]_c$ used as an indirect measurement of ATP consumption.

The protonophore CCCP (10 μ M) was perfused at the end of experiments as a control, to collapse reliably the mitochondrial membrane potential, and with this any ATP generation via cellular respiration (demonstrated as a rise in $[Mg^{2+}]_c$). The importance of supplementary ATP to the cell, during toxic stimuli, is shown in this set of results as there is no additional rise in $[Mg^{2+}]_c$ in response to CCCP when 0mM ATP is present in the internal solution – indicating the cell has already consumed its ATP reserve with simultaneous inhibition of ATP production, a situation liable to result in necrosis.

Toxicity of transformed secretagogue- FAEE elicited Ca^{2+} signals depends on FAEE hydrolysis and mitochondrial impairment

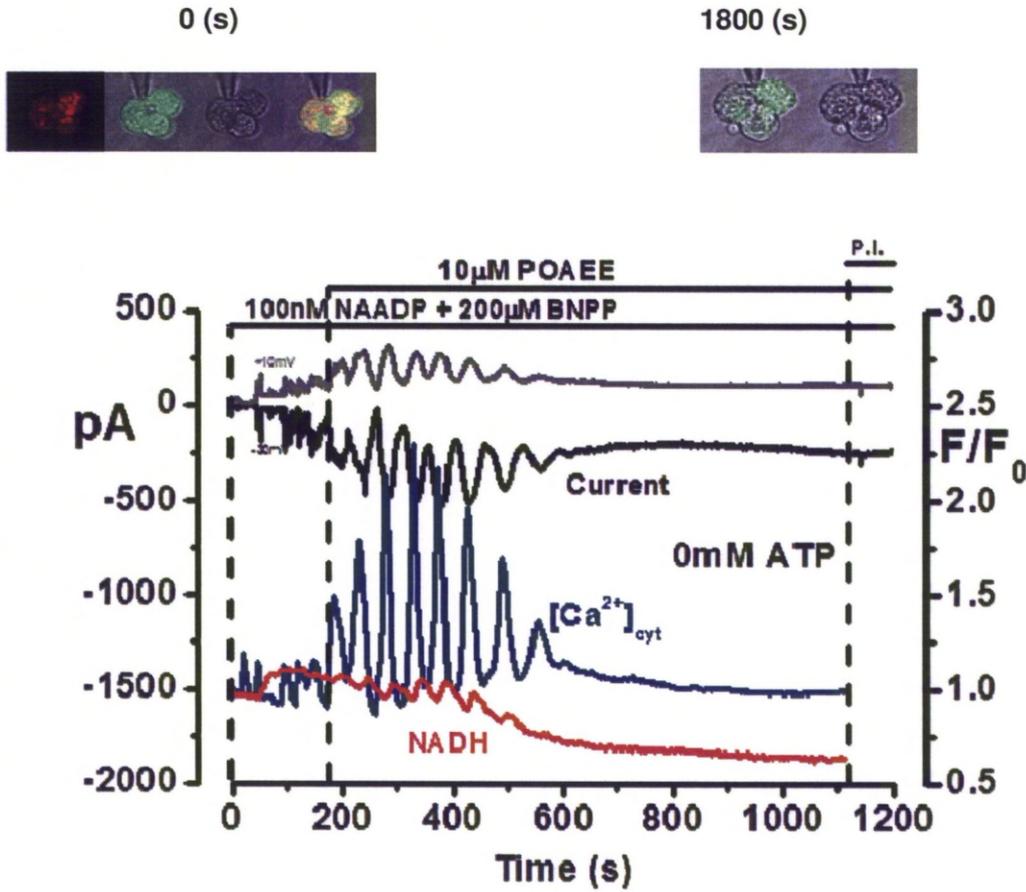
FAEEs accumulate within mitochondria where hydrolysis releases FAs that can inhibit β oxidation, uncouple oxidative phosphorylation, reduce $\Delta\psi_m$ and diminish ATP production (Criddle et al, 2006). Although 10 μM POAEE was found not to damage cells, palmitoleic acid (POA) released by hydrolysis of POAEE at this concentration might induce a modest degree of mitochondrial compromise that could render the mitochondrion less able to withstand sustained elevations of $[\text{Ca}^{2+}]_c$. (Criddle et al, 2006). Therefore bis-(4-nitrophenyl) phosphate (BNPP, 200 μM) was applied to cells before stimulation, as a known inhibitor of FA hydrolysis that prevents toxicity from high concentrations (100 μM) of FAEEs in unstimulated cells. When 10 μM POAEE was then added after the start of stimulation with either ACh, CCK, IP_3 or NAADP, transformation was again immediate, of large amplitude and global, but was more rapidly and repeatedly cleared from the cytosol, with well-maintained NADH signals and no detectable PI uptake (Figure 4.10). The signals tended to lessen in time, suggesting possible saturation of second messenger receptors with Ca^{2+} . The experimental data summary is shown in Table 4.5. The data is consistent with a role for FAs in Ca^{2+} toxicity from FAEE-induced transformation, brought about by modest mitochondrial compromise that renders cells less able to cope with Ca^{2+} overload.

Figure 4.10 (A)



Normal physiological elevations of $[Ca^{2+}]_c$ induced by physiological concentrations of IP_3 ($1.5\mu M$) are transformed into global elevations of $[Ca^{2+}]_c$ with the perfusion of a low concentration of POAEE when 0 mM ATP is present. Previously this would have resulted in cell injury, but here, perfusion with the hydrolase inhibitor BNPP ($200\mu M$) has prevented cellular necrosis. Note the NADH production by the functioning mitochondria in response to the large elevations of $[Ca^{2+}]_c$ indicating ATP production. The upper panels demonstrate no cellular necrosis at the end of the experiment.

Figure 4.10 (B)



Normal physiological elevations of $[Ca^{2+}]_c$ induced by 100nM NAADP are transformed into global elevations of $[Ca^{2+}]_c$ with the perfusion of a low concentration of POAEE when 0mM ATP is present. However, here, perfusion with the hydrolase inhibitor BNPP (200μM) has prevented cellular necrosis which occurs in its absence. Note the NADH production (red line) by the functioning mitochondria in response to the large elevations of $[Ca^{2+}]_c$.

Table 4.5

Stimulus/ Toxin 0mM ATP + 200µM BNPP	Stimulus/ Toxin 0mM ATP	Necrosis	No Necrosis
1pM CCK / 10µM POAEE	-	1/7 Cells	6/7 Cells
-	1pM CCK / 10µM POAEE	7/7 Cells	0/7 Cells
20nM ACh / 10µM POAEE	-	0/5 Cells	5/5 Cells
-	20nM ACh / 10µM POAEE	6/6 Cells	0/6 Cells
50nM NAADP / 10µM POAEE	-	2/7 Cells	5/7 Cells
-	50nM NAADP / 10µM POAEE	8/9 Cells	1/9 Cells
1µM IP ₃ / 10µM POAEE	-	0/7 Cells	7/7 Cells
-	1µM IP ₃ / 10µM POAEE	8/9 Cells	1/9 Cells

The secretagogues/ second messenger and ethanol metabolite combination, results in toxic calcium signals when supplementary ATP is absent. The pathological combinations were tested for cell viability with or without external perfusion with the hydrolase inhibitor BNPP. When BNPP was present, mitochondrial impairment is avoided and no cell injury results.

High dose bile acids alone induce cellular toxicity by excessive $[Ca^{2+}]_c$ release and mitochondrial ATP production failure

The pathological mechanism of action of bile salts on pancreatic acinar cells is controversial. Prior results have demonstrated a sustained global cytosolic Ca^{2+} rise in response to supramaximal bile salt stimulation applied directly, a situation that is unlikely to be persistently present in the clinical situation – but not impossible (Voronina et al, 2002). The Ca^{2+} release is mediated via IP_3 receptors and ryanodine receptors as demonstrated here and previously (Voronina et al, 2002, Gerasimenko et al, 2006). But whether the excessive Ca^{2+} release by itself triggers the chain of events leading to cellular compromise is uncertain.

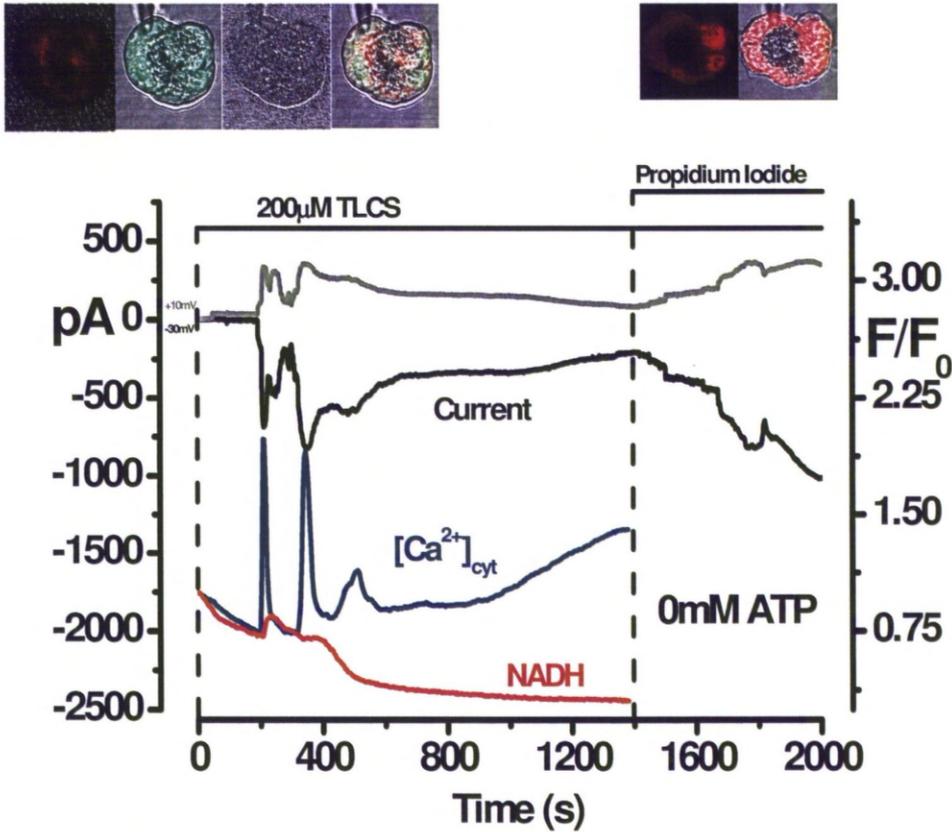
It has been hypothesized that the bile salts directly inhibit the SERCA pumps (Kim et al, 2002) eliciting the sustained global Ca^{2+} rise as the cell is unable to clear the excessive build up of Ca^{2+} which leads to premature trypsinogen activation, activation of cytokines and nuclear factor kappa B (NF- κ B) ultimately leading to morphological features of acute pancreatitis and cell death.

To test this, the same electrophysiological set up employed previously with simultaneous confocal imaging is ideal to test whether it is inhibit of the Serca pumps directly, or actual depletion of ATP leading to failure of ATP dependent Ca^{2+} pumps which leads to excessive accumulation of the cytosolic Ca^{2+} and subsequent cell injury. When the pancreatic acinar cells are subjected to perfusion with a high dose of bile acids (200 μ M TLCS), there is a sustained rise in cytosolic Ca^{2+} in cells that have 0mM supplementary ATP in the patch pipette. This culminates in cell necrosis (figure 4.11) but does not give us any insight into whether it is the toxic Ca^{2+} release, ATP rundown

and inhibition or direct action on the Serca pumps which is the major factor in providing the sustained pathological Ca^{2+} rise.

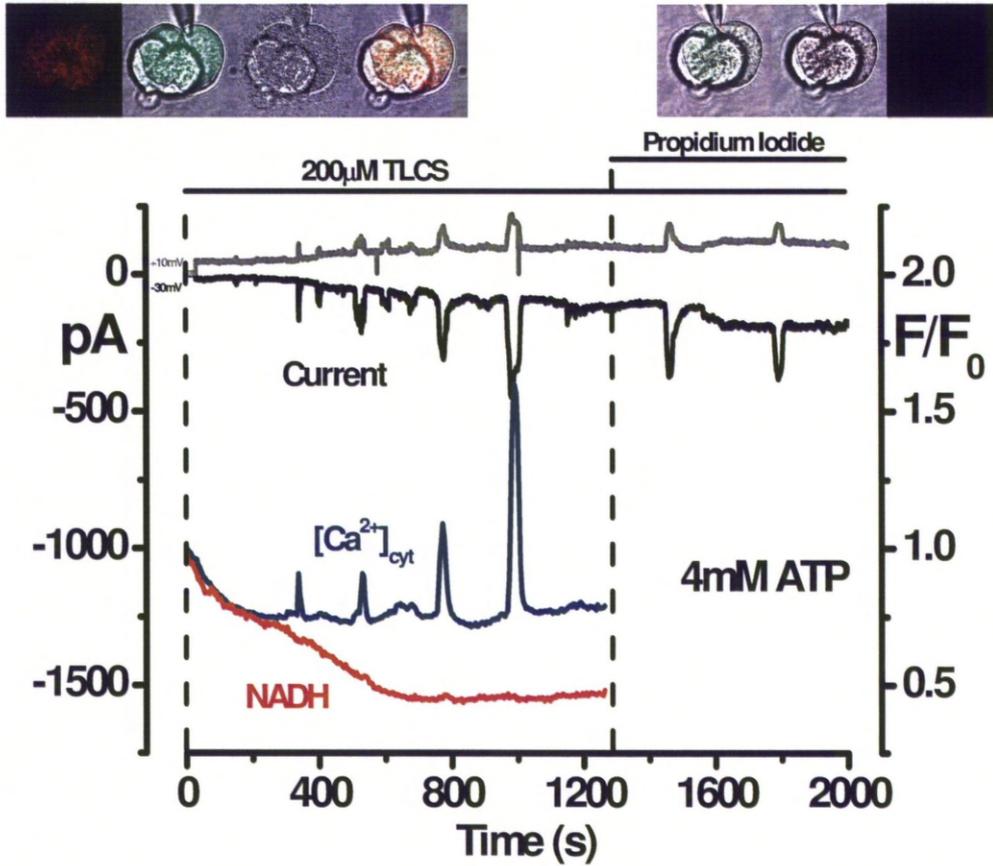
Therefore, with the addition of supplementary ATP (4mM) to the patch pipette, the ATP dependent Ca^{2+} extrusion pumps are able to continue to pump Ca^{2+} out of the cytosol preventing a sustained, global pathological rise (figure 4.11 B+C) (Table 4.6). This suggests that it is the actual ATP depletion and lack of production that is responsible for the SERCA pumps and PMCA pump failure culminating in cell injury, in much the same process as demonstrated with the secretagogue/ low dose toxin combination demonstrated earlier in this chapter.

Figure 4.11(A)



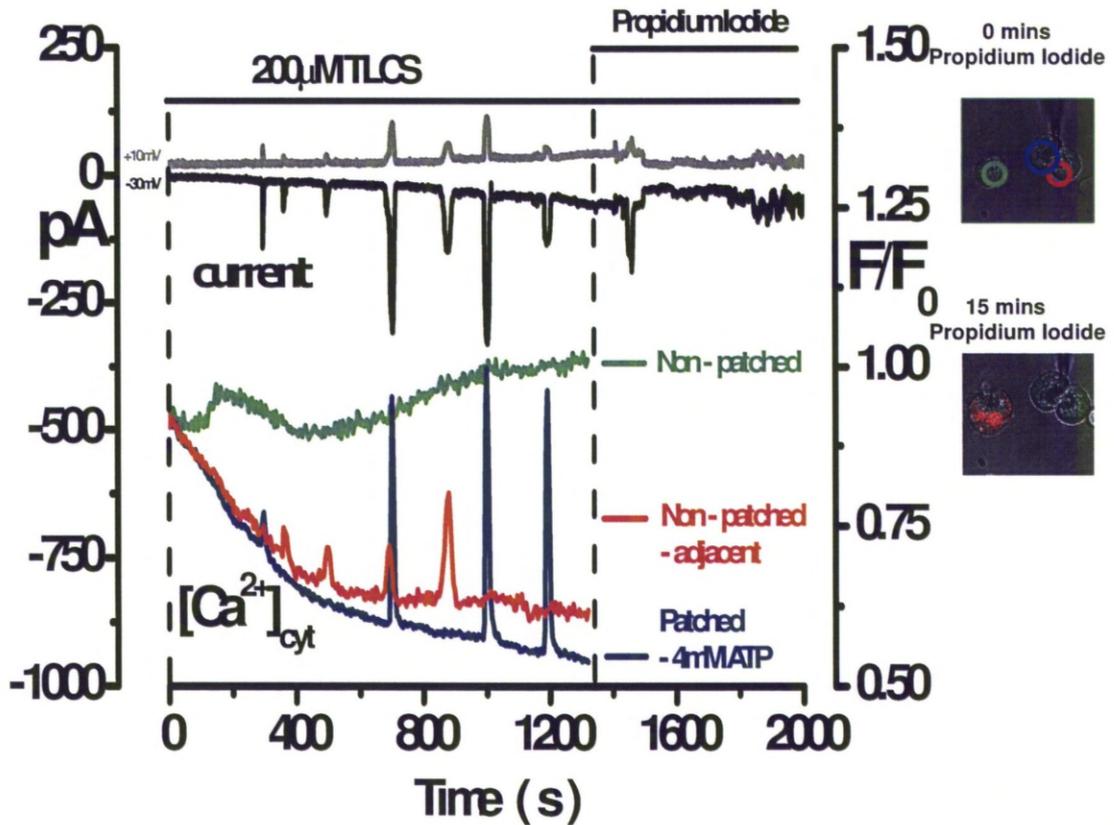
High concentrations of TLCS (200μM) induce a sustained rise in $[Ca^{2+}]_c$ in the patched cell with no supplementary ATP. Note the NADH initially rises in response to the large oscillations but collapses in tandem with a sustained rise in $[Ca^{2+}]_c$ as complete ATP depletion occurs. The net result is cellular necrosis as can be seen in the top panels with positive staining with propidium iodide in both the cell attached to the patch pipette and the adjacent cell.

Figure 4.11 (B)



High concentrations of TLCS (200μM) induce a global rise in $[Ca^{2+}]_c$ but is not sustained in the patched cell due to supplementary ATP allowing the ATP dependent Ca^{2+} pumps to continue the function of extruding Ca^{2+} from the cytosol. Consequently, no cellular necrosis is observed with propidium iodide in the patched cell or adjacent cell – presumably due to protection conferred by the patched cell with supplementary ATP.

Figure 4.11 (C)



High concentrations of TLCS ($200\mu\text{M}$) induce a sustained rise in $[Ca^{2+}]_c$ in the non-patched cell versus the patched cell and its doublet partner in which large oscillations occur. The supplementary ATP provided by the patch pipette solution protects the cell and its neighbour against necrosis which results in the isolated non-patched cell. This indicates a sufficient absence of ATP in the non-patched cell results in a sustained rise in $[Ca^{2+}]_c$ with cell necrosis the terminal result.

Table 4.6

High Dose Toxin	0mM ATP	4mM ATP	Global $[Ca^{2+}]_c$ rise	Necrosis	No Necrosis
200 μ M TLCS	7 Cells	-	7/7 Cells	6/7 Cells	1/7 Cells
200 μ M TLCS	-	7 Cells	7/7 Cells	0/7 Cells	7/7 Cells

Perfusion with high concentrations of bile acids induce global $[Ca^{2+}]_c$ rises and cellular necrosis when supplementary ATP is absent. This is avoided with the delivery of supplementary ATP to the cell via the pipette.

Discussion

Here a new mechanism for necrotic cell death caused by excessive Ca^{2+} release into the cytosol, through second messenger receptor Ca^{2+} channels from intracellular stores is identified. The new data show that these channels are critical targets for low μM (physiological) concentrations of specific toxins which dramatically increase and prolong their open-state probabilities at the time of binding of their respective second messengers. The consequent sustained cytosolic Ca^{2+} rise overloads mitochondria with Ca^{2+} with inhibition of mitochondrial ATP production, creating a vicious circle in which SERCA and PMCA clearance of cytosolic Ca^{2+} is impaired, further mitochondrial injury is sustained, and necrotic cell death inevitable. Toxic transformation is the clear cause but not result of ATP depletion, since low cytosolic $[\text{ATP}]_c$ inhibits second messenger receptor Ca^{2+} release, and SERCA pump inhibition does not directly affect IP_3 -, cADP- or NAADP-evoked Ca^{2+} signals. Although, the extent of toxic transformation is dependent on Ca^{2+} store refilling from Ca^{2+} outside the cell. Specific second messenger receptor blockade demonstrated that toxic transformation was due completely to release from their Ca^{2+} channels, rather than any direct effect of toxins on Ca^{2+} entry mechanisms.

Mitochondria respond rapidly to $[\text{Ca}^{2+}]_c$ elevations by Ca^{2+} uptake through the high affinity uniporter, driven by an electrochemical gradient of protons (measured as mitochondrial membrane potential, $\Delta\psi_m$) of up to 200 mV, integrating cellular ATP requirements, which serves to buffer and extrude Ca^{2+} via a mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger to end the signal. Sustained, generalised elevations of $[\text{Ca}^{2+}]_c$ overload mitochondria and dissipate $\Delta\psi_m$, the collapse of which was so marked after toxic

transformation in the experiments that we found the protonophore CCCP to induce no further drop in ATP production.

The different patterns of second messenger receptor transformation specific to the two different toxins are consistent with different molecular interactions, most simply explained by different patterns of binding, perhaps removing inhibitory effects of Ca^{2+} on Ca^{2+} channel opening. The effect of POAEE on IP_3 R is determined by its ethanol ester group, without which POA ($>100 \mu\text{M}$) does not induce spontaneous Ca^{2+} release from IP_3 R (Criddle et al, 2006). Although POAEE was found to transform NAADP-evoked Ca^{2+} signals, this could have occurred due to CICR via IP_3 R with an increased open-state probability from POAEE. NAADP-evoked Ca^{2+} signalling is known to depend on local recruitment of IP_3 R and RyR (Cancela et al, 2002), which would account for the inhibition of NAADP-evoked Ca^{2+} signals and inhibition of their transformation by POAEE upon application of the IP_3 R inhibitor caffeine.

On the other hand, bile salts ($>100 \mu\text{M}$) induce Ca^{2+} release from RyR's as well as IP_3 R, the most potent being TLCS (Voronina et al, 2002). TLCS makes up to 5% of human bile, that can spill into the systemic circulation in cholestatic jaundice. Thus transformation of cADPR-evoked as well as IP_3 evoked Ca^{2+} signals by TLCS may account for toxicity in the pancreas. The summative effects of the physiologically likely concentrations of toxins on simultaneous physiological G-protein-linked receptor stimulation is more likely than hyperstimulation to account for clinical pancreatitis, where second messenger release and bile salts as well as FAEE's have the same Ca^{2+} releasing properties. Substantial data implicate IP_3 R and RyR's as contributors to cell

injury and necrosis, but only now have we found transformed Ca^{2+} release from these receptors to be the sole, direct cause of unprogrammed, necrotic cell death.

Chapter 5

Conclusions

In most experimental models, acute pancreatitis is induced by methods of supramaximal stimulation of some form. Although these models have provided genuine insight into the possible and probable pathophysiological mechanisms leading to the clinical manifestation of the disease, one can always criticise the methodology, as supramaximal stimulation would very rarely, if ever, happen in the clinical situation. In the last chapter this point has been answered by mimicking what may well happen in the clinical situation. For example, a patient presenting to the Emergency Department with abdominal pain and vomiting (secondary to gallstone induced pancreatitis) will most likely have been eating and drinking normally until the onset of symptoms. From this we can speculate that there is a possibility that the pancreas and more specifically, the pancreatic acinar cells will have been subjected to neurotransmitter, hormonal and bile salt stimulation (secondary to bile reflux occurring with gallstone blockade at the Ampulla of Vater), at the same time.

What is demonstrated experimentally is an attempt to mimick this situation with prior stimulation with physiological neurotransmitters, hormones etc. and then stimulate with concentrations of toxins, such as bile salts or FAEE's, which are more likely to be achieved consistently in the clinical situation which can account for the increasing incidence in acute pancreatitis in the Western world.

The same principle is applied to the rapidly increasing incidence of acute pancreatitis secondary to excessive ethanol consumption. Previous data from our laboratory has demonstrated that ethanol itself, and the oxidative metabolite acetaldehyde has little effect on Ca^{2+} signalling in acutely isolated pancreatic acinar cells, but the non-oxidative Fatty Acid Ethyl Esters are potentially hugely damaging to the cells (Criddle et al,

2004). This is actually common sense, as every person who has a large intake of alcohol once in a while does not suffer from acute pancreatitis, presumably as the liver can breakdown ethanol in the preferred way oxidatively using alcohol dehydrogenase and acetylaldehyde being the final product (Criddle et al, 2004). However, the patients who persistently have heavy alcohol intake may have to metabolise the ethanol via the non-oxidative pathway producing FAEE's. Coupled with neurotransmitter and hormonal stimulation, and data suggesting that acinar cells subjected to long term alcohol exposure are more sensitive to receptor stimulation (Gukovskaya et al, 2004, Kubisch et al, 2004), the acinar cell is stimulated in a similar fashion to what has been attempted here giving us greater insight into the pathophysiology of ethanol induced acute pancreatitis.

Bile salts with second messengers/ secretagogues

The data gained from the experiments using the second messengers in the internal solution, verified with the use of secretagogues, (knowledge of their mechanism of action gained from previous work) (Cancela et al, 2002, Voronina et al 2002, Gerasimenko et al, 2006), is very valuable when identifying possible therapeutic strategies in the clinical situation in the future. The demonstration that the bile salts cause Ca^{2+} release via two different pathways at least, namely the IP_3 receptor and the ryanodine receptor, identifies potential targets to halt the disease process in the very earliest stage before the progression to severe acute pancreatitis with associated sequelae.

Non-oxidative ethanol metabolites with second messengers/

secretagogues

The mechanisms of action have been demonstrated in this work that give insight into how the FAEE's cause cytotoxic Ca^{2+} release. Ca^{2+} release via the IP_3 receptors has recently been described by our laboratory (Park et al, 2002) and has been confirmed (Criddle et al 2004, 2006), but the release via the second messenger NAADP is a new finding and potentially very significant. Debate still rages on whether NAADP causes Ca^{2+} release via the ryanodine receptor or has a, as yet unidentified, specific receptor unique to NAADP (Gallione et al, 2006, Cosker et al, 2010). Whichever the case, NAADP is an extremely potent Ca^{2+} release second messenger vital in the pathological Ca^{2+} release in response to FAEE's.

Toxic intracellular Ca^{2+} elevation, ATP depletion and cell injury

The initial stages of the project had identified the mechanisms of excessive intracellular Ca^{2+} in response to the low dose toxin stimulation with the physiological second messenger/ secretagogue combinations. This, however, does not explain entirely the cell injury that may or may not result as a consequence of the elevated Ca^{2+} levels. Although, work is ongoing into how a sustained elevation of cytosolic Ca^{2+} results in morphological features of acute pancreatitis, the precise mechanisms are unknown. The most severe and potentially lethal form of acute pancreatitis is necrotizing acute pancreatitis. In the experiments demonstrated above, the assessment for necrosis and cellular energetics in the process is a model which could describe what is actually happening in the clinical situation. The latest experimental data has demonstrated that

a persistent toxic rise in Ca^{2+} is able to open the mitochondrial permeability transition pore (MPTP) (Gerasimenko et al, 2009) which disables the mitochondrial ability to efficiently produce ATP via the Krebs cycle and the electron transport chain (ETC) is jeopardised through unchecked entry of cations down the electrochemical gradient. The mitochondrion is also subject to osmotic swelling as lower molecular weight ions and proteins are able to enter the cristae of the mitochondrial through osmosis with further disabling of the mitochondrial ability to produce ATP the result (Criddle et al, 2007, Gerasimenko et al, 2009)

Previous data has suggested that the high dose bile salts (TLCS) cause a raised cytotoxic elevation in intracellular Ca^{2+} by inhibiting the SERCA pumps themselves directly, therefore inhibiting the replenishment of Ca^{2+} back into the ER after Ca^{2+} release and providing the prolonged elevation in cytosolic Ca^{2+} and subsequent sequelae (Kim et al, 2002). The results demonstrated here, however, support the theory that the SERCA pumps are indeed faulty, but it is because of general inhibition of ATP dependent Ca^{2+} pumps, not only the SERCA pumps, by inhibiting the production of, and massive consumption of available ATP in the cell.

This is demonstrated most effectively with high doses of bile salts perfused alone in which cells which are patch clamped with no supplementary ATP and non patched cells undergoing necrosis following a massive sustained intracellular cytosolic Ca^{2+} release. But, when ATP is added to the internal contents of the cell, necrosis is avoided but not in cells that are separate from the patched clamped cell or adjacent ones. When analysed in greater detail, the real time intracellular Ca^{2+} events demonstrate that the cells in which supplementary ATP is available are subject to large global Ca^{2+} release

but the cell is able to extrude the large loads of cytosolic Ca^{2+} back in to ER via the SERCA pumps and outside the cell via PMCAs, because ATP is available in large quantities so that the pumps keep working efficiently avoiding a sustained toxic rise in cytosolic Ca^{2+} leading to cell injury.

When no supplementary ATP is available and in non-patchclamped cells, the ATP available is consumed very quickly due to the massive Ca^{2+} release which causes inhibition of the ATP dependent Ca^{2+} extrusion pumps (SERCA and PMCA's) leading to the toxic build up of cytosolic Ca^{2+} . This in turn leads to mitochondrial membrane depolarisation and further inhibition of ATP production, which in turn leads to further disabling of the Ca^{2+} ATPase pumps, calcium induced calcium release (CICR) and the probable switching on of the store operated channels (SOC's). This chain of events leads to a sustained elevated concentration of cytosolic Ca^{2+} , switching on mechanisms such as cell death pathways (which in the absence of ATP cause necrosis), cytokine production, premature intracellular trypsinogen activation etc. leading to acute pancreatitis (Raraty et al, 2000, Sutton et al, 2003).

In the more physiologically relevant situation described above, the same mechanisms and process of events probably occur but at a slower rate and so it is a general build up of cytosolic Ca^{2+} leading to the events culminating in cell necrosis. This is supported by the investigation using indirect measure of ATP consumption using Magnesium Green fluorescence, in which ATP depletion

occurs over the time course with excessive Ca^{2+} entry unresponsive to protonophore treatment.

This experimental set up also attempts to imitate the possible course of events clinically as the patient who presents with gallstone pancreatitis develops pancreatic necrosis over a period of time – probably due to prolonged stimulation and contact with bile acids due to the blockade of the pancreatic duct. This theory is supported by two major observations:

- 1) Enhanced Computed Tomography of the pancreas on day 1 of onset of symptoms shows an inflamed pancreas whereas, usually, it is not until day 5 or beyond in which mass pancreatic necrosis becomes evident,
- 2) Early endoscopic removal of gallstone blockade is performed as it shows significantly better prognostic outcome compared to delay (Neoptolemos et al, 1988, Folsch et al, 1997).

Previously experimental data (Criddle et al 2004), demonstrates how important fatty acid ethyl esters may be in the pathogenesis of acute pancreatitis of ethanol aetiology by causing toxic elevation of cytosolic Ca^{2+} in pancreatic acinar cells, ie. through activation of inositol trisphosphate receptors and inhibition of ATP production that results in SERCA and PMCA pump failure.

Further insight into the pathophysiological events has also been identified (Criddle et al, 2006). Supramaximal concentrations of FAEE's (100 μM) dissolved in large quantities of ethanol (850mM) were first used to allow experimentation to take place. In clinical practice, although possible, it would be rare to attain such large serum and pancreatic

ductal quantities of FAEE's and certainly impossible to gain serum quantities of ethanol to that extent (Criddle et al, 2004, 2006).

So the beauty of the results demonstrated in chapter 4 is that the mechanisms of cell injury are again demonstrated and a new mechanism of Ca^{2+} release (NAADP) is shown. But, more importantly, the concentrations of FAEE'S and ethanol used are very likely to be able to be representative in patients presenting with ethanol induced acute pancreatitis.

Although it seems that the end point of a sustained pathological rise in cytosolic Ca^{2+} is a major trigger for the train of events that lead to acute pancreatitis, the preceding mechanisms are subtly different between bile acid induced and FAEE induced pancreatic acinar cell injury.

It seems that the excessive cytosolic Ca^{2+} release and inhibition of mitochondrial function are independent of each other, the former responsible by the FAEE itself whereas mitochondrial function is directly inhibited by the fatty acid, formed by hydrolysis inside the cell located around the mitochondria (Criddle et al, 2006).

Blockade of the IP_3 receptor failed to prevent cellular necrosis in studies looking at the supramaximal doses of the fatty acid ethyl esters - presumably due to the high concentration of fatty acid produced by intracellular hydrolysis and collapse of subsequent mitochondrial function (Criddle et al, 2006). In the more clinically relevant situation though, cell necrosis is avoided probably due to the lowering of the energy demands of the acinar cell without global Ca^{2+} release and a much lower concentration of fatty acids made in response to the lower concentration of fatty acid ethyl esters. Therefore IP_3 receptor blockade may be an attractive therapeutic target in the future

(figure 5.1), and indeed it is suggested that large coffee drinkers are less susceptible to severe acute pancreatitis precipitated by ethanol (Sutton et al, 2006).

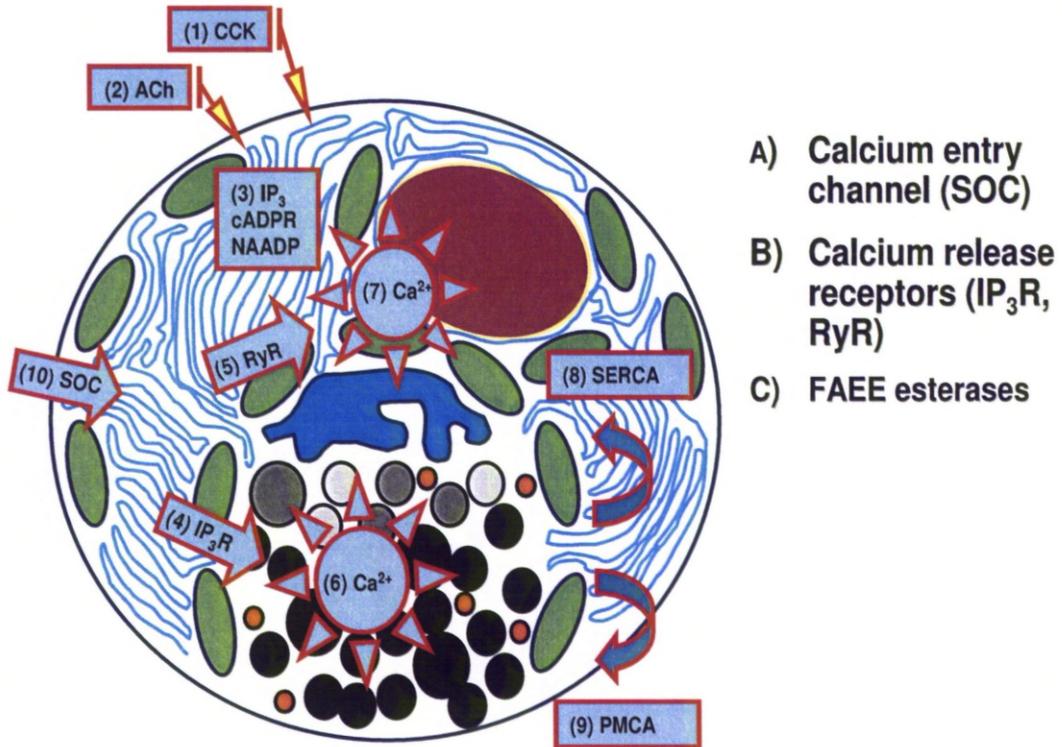
When hydrolysis inhibition is achieved, the cells are still able to produce ATP in response to the large global Ca^{2+} release allowing cytosolic Ca^{2+} extrusion and maintenance of basal cytosolic Ca^{2+} levels. This is of major importance as mentioned earlier; it is the sustained elevated level of cytosolic Ca^{2+} that is the crucial factor in the development of acute pancreatitis.

Another important, potential future therapeutic target is the store operated channels. A large amount of work has allowed greater understanding in this area recently and results are promising if applied to the pancreas. In work detailed in chapter 4, as well as by several authors before, the importance of external Ca^{2+} has been stressed. In many studies acute pancreatitis can be avoided in the absence of external Ca^{2+} (Raraty et al, 2000). This, of course, is a clinical impossibility but the mechanism of why this is important is becoming more apparent.

Store operated Calcium channels are located on the plasma membrane and are activated by large sustained rises in Ca^{2+} , causing a further influx of Ca^{2+} into the cell further increasing the toxic cytosolic Ca^{2+} load. If a SOC antagonist were to become available in the future it may be an attractive therapy in the early course of the disease process (figure 5.1).

Figure 5.1

Therapeutic targets



- A) Calcium entry channel (SOC)
- B) Calcium release receptors (IP_3R , RyR)
- C) FAEE esterases

Figure 5.1

Potential therapeutic targets are demonstrated in the schematic of the pancreatic acinar cell:

A) Blockade of Ca^{2+} entry through Store Operated Channels antagonism may halt a sustained intracellular Ca^{2+} elevation limiting the exposure of the mitochondria to the toxic Ca^{2+} load.

B) Blockade of Ca^{2+} release from the intracellular stores via second messenger release channels antagonism (IP_3 R and RYR). Such agents such as caffeine and dantolene may have a role (IP_3 R and RYR antagonist respectively)

C) FAEE esterases inhibit intracellular formation of fatty acids. This may protect the mitochondria and maintain the membrane potential so ATP is available for the Ca^{2+} ATPase pumps and could be of therapeutic value in ethanol induced acute pancreatitis. Although the possible targets are highlighted separately above, it may be that a combination of targets may be used in an attempt to prevent a toxic cytosolic Ca^{2+} load and deficit ATP supply.

Pancreatic Acinar Cell death – apoptosis or necrosis?

So far we have concentrated on how to treat the disordered intracellular calcium signalling in order to avoid the pathological downstream events leading to cellular necrosis, but we must also explore other avenues. Severe necrotizing pancreatitis can be avoided if the pancreatic acinar cell's fate were to be directed via the apoptotic route rather than by necrosis. This is because necrosis is an energy consuming process in which the inflammatory cascade is activated both locally and systemically.

It is currently uncertain whether reactive oxygen species generation in the pancreatic acinar cell in response to toxic stimuli is noxious or not and is currently controversial (Mukerjee et al, 2007). We know that disordered calcium signalling within the acinar cell in response to bile acids at least, generate cell death via apoptosis through the intrinsic pathway (Baumgartner et al, 2008). Mechanisms are also in place to generate caspase mediated apoptosis via the non- classical pathway. It is likely that cell death via necrosis occurs in response to the strength the disordered intracellular Ca^{2+} elevation and the length of time of which the toxic insult persists (Baumgartner et al, 2008, Gerasimenko et al, 2009).

However, if there were mechanisms in which the intracellular signalling pathways could be manipulated then the pancreatic acinar cell may be persuaded to enter an apoptotic rather than a necrotic cell death pathway. In this vein, more studies on the role of ROS in cell injury may provide a therapeutic target to prevent mass cellular destruction early

in the disease course. To this end, if patients suffering from acute pancreatitis can be limited to a 'mild' self-limiting attack then the burden, both in terms of morbidity/mortality and financial cost would be vastly improved and indeed acute pancreatitis would not be a 'feared' disease, both by the patient and clinician.

Of course, possibly the most important set of results demonstrated here are those attaining to human pancreatic acinar cells. Until now, a simple question that hung over the field of Pancreatology was whether human pancreatic cells actually responded to secretagogues like murine cells, and therefore, realistically what conclusions can be drawn and taken forward into the world of clinical practice (Ji et al, 2001, 2002).

A perfectly reasonable question as before the work presented here, there was no evidence of human acinar cell response to cholecystokinin, and indeed conflicting evidence suggesting that the cells only respond to neural stimulation.

But, the demonstration of functional CCK receptors in human pancreatic acinar cells defines a major parallel underlining the relevance and significance of in vitro and in vivo rodent work that examines the role of CCK in pancreatic exocrine function and disease (Peterson et al, 2008, Saluja et al, 2007, Jensen et al, 2002, Pandol et al, 2003, Gukovsky et al, 2004, Husain et al 2005).

The work has shed new light into the field of clinical Pancreatology opening doors for possible clinical trials based on animal experimental design – a large step forward if we, as a profession, are to make inroads into developing useful treatment strategies in the prevention of severe necrotizing acute pancreatitis and associated morbidity and mortality.

Reference List

Alberts, B; Johnson A, Lewis J, Raff M, Roberts M, Walter P. 1994. Molecular Biology of the Cell. New York, Garland Publishing Inc.

Ammann RW, Heitz PU, Kloppel G. Course of alcoholic chronic pancreatitis: a prospective clinicomorphological long-term study. *Gastroenterology* 1996;111:224-31.

Armstrong JS, Yang H, Duan W, and Whiteman M. Cytochrome bc1 regulates the mitochondrial permeability transition by two distinct pathways. *Journal of Biological Chemistry* 2004;279:48, 50420-50428.

Ashby MC, Tepikin AV. Polarised Calcium and Calmodulin Signalling in Secretory Epithelia. *Physiol Rev* 2002; 82: 701-734

Ashby MC, Craske M, Park MK, Gerasimenko OV, Burgoune RD, Peterson OH, Tepikin AV. Localised calcium uncaging reveals polarised distribution of calcium sensitive calcium release sites: mechanism of unidirectional calcium waves. *J Cell Biol* 2002; 158: 283 – 92

Ashby MC, Camello-Almaraz C, Gerasimenko OV, Peterson OH, Tepikin AV. Long distance communication between muscarinic receptors and calcium release channels revealed by carbachol uncaging in cell attached patch pipette. *Jour Biol Chem* 2003; 23: 20860 – 20864

Babior BM. Oxygen-dependent microbial killing by phagocytes. *N Engl J Med* 1978; 298: 659-668

Baker PF, Knight DE. Calcium-dependent exocytosis in bovine adrenal medullary cells with leaky plasma membranes. *Nature* 1978;276:620-622.

Bartholomew C. Acute scorpion pancreatitis in Trinidad. *British Medical Journal* 1970; 1: 666-668

Baumgartner HK, Gerasimenko JV, Thorne C, Ashurst LH, Barrow SL, Chvanov MA, Gillies S, Criddle DN, Tepikin AV, Petersen OH, Sutton R, Watson AJ, Gerasimenko OV. Caspase-8-mediated apoptosis induced by oxidative stress is independent of the intrinsic pathway and dependent on cathepsins. *Am J Physiol Gastrointest Liver Physiol.* 2007;293(1):G296-307

Baumgartner HK, Gerasimenko JV, Thorne C, Ferdek P, Pozzan T, Tepikin AV, Petersen OH, Sutton R, Watson AJ, Gerasimenko OV. Calcium elevation in mitochondria is the main Ca²⁺ requirement for mitochondrial permeability transition pore (mPTP) opening. *J Biol Chem*. 2009;284(31):20796-803

Beger HG, Buchler M, Bittner R, Block S, Nevalainen T, Roscher R. Necrosectomy and postoperative local lavage in necrotizing pancreatitis. *Br J Surg*. 1988;75(3):207-12.

Belan PV, Gerasimenko OV, Tepikin AV, Peterson OH. Localisation of calcium extrusion sites in pancreatic acinar cells. *J Biol Chem* 1996; 271: 7615-9

Benifla M, Weizman Z. Acute pancreatitis in childhood: analysis of literature data. *J Clin Gastroenterol*. 2003;37(2):169-72.

Benson JR, Jatoi I, Keisch M, Esteva FJ, Makris A, Jordan VC. Early breast cancer. *Lancet*. 2009;373(9673):1463-79.

Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. *Nat Rev Mol Cell Biol* 2000; 1: 11-21

Beutner G, Rück A, Riede B, Brdiczka D.. Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. *Biochimica et Biophysica Acta*, 1998,1368, Issue 1, Pages 7-18.

Bezprozvanny I. The inositol 1,4,5-trisphosphate receptors. *Cell Calcium* 2005;38:261-272.

Bhatia M, Brady M, Zagorski J, Christmas SE, Campbell F, Neoptolemos JP, Slavin J. Treatment with neutralising antibody against cytokine induced neutrophils chemo attractant (CINC) protects rats against pancreatitis associated lung injury. *Gut* 2000; 47: 838-44

Bhatia M, Brady M, Kang YK, Costello E, Newton DJ, Christmas SE, Neoptolemos JP, Slavin J. MCP-1 but not CINC synthesis is increased in rat pancreatic acini in response to caerulein hyperstimulation. *Am J Physiol Gastrointest Liver Physiol* 2002; 282: G77 - 85

Bhatia M. Apoptosis versus necrosis in acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol*. 2004;286(2):G189-96

Blinman TA, Gurovsky I, Mouria M, Zaninovic V, Livingston E, Pandol SJ, Gukovskaya AS. Activation of pancreatic acinar cells on isolation from tissue: cytokine up regulation via p38 MAP kinase. *Am J Cell Physiol* 2000; 279: C1993-003

Bolender, RP. Stereological study of pancreatic exocrine cells. *Adv. Cytopharmacol.* (1974). 2. 99-106

Bradley EL 3rd, Allen K. A prospective longitudinal study of observation versus surgical intervention in the management of necrotizing pancreatitis. *Am J Surg.* 1991;161(1):19-24;

Bradley EL 3rd. A fifteen year experience with open drainage for infected pancreatic necrosis. *Surg Gynecol Obstet.* 1993;177(3):215-22.

Bray JJ, Cragg PA, Macknight ADC, Mills RG, Taylor DW. *Lecture notes in physiology*, third edition 1994, Blackwell Science, 298 – 302

Buchler MW, Gloor B, Muller CA, Friess H, Seiler CA, Uhl W. Acute necrotizing pancreatitis: treatment strategy according to the status of infection. *Ann Surg.* 2000;232(5):619-26

Büki A, Okonkwo DO, Wang KKW, and Povlishock JT. Cytochrome c release and caspase activation in traumatic axonal injury. *Journal of Neuroscience*. 2000. Vol 20, Issue 8,2825-2834.

Buter A, Imrie CW, Carter CR, Evans S, McKay CJ. Dynamic nature of early organ dysfunction determines outcome in acute pancreatitis. *Br J Surg*. 2002;89(3):298-302.

Byung Kyu Park, Jae Bock Chung, Jin Heon Lee, Jeong Hun Suh, Seung Woo Park, Si Young Song, Hyeyoung Kim, Kyung Hwan Kim, Jin Kyung Kang. Role of oxygen free radicals in patients with acute pancreatitis *World J Gastroenterol* 2003;9(10):2266-2269

Camello C, Lomax R, Peterson OH, Tepikin AV. Calcium leak from the intracellular stores – the enigma of calcium signalling. *Cell Calcium* 2002; 32: 355-361

Campos-Toimil M, Edwardson JM, Thomas P. Real-time studies of zymogen granule exocytosis in intact rat pancreatic acinar cells. *J Physiol* 2000;528:317-326.

Cancela JM, Gerasimenko OV, Gerasimenko JV, Tepikin AV, Peterson OH. Two different but converging messenger pathways to intracellular calcium release: the roles of nicotinic acid adenine dinucleotide phosphate, cyclic ADP-ribose and inositol triphosphate. *EMBO J* 2000; 19: 2549 -57

Cancela JM, Van Coppenolle F, Galione A, Tepikin AV, Peterson OH. Transformation of local calcium spikes to global calcium transients: the combinatorial roles of multiple calcium releasing messengers. *EMBO J* 2002; 21: 909 -19

Cancela JM, Petersen OH. Regulation of intracellular Ca^{2+} stores by multiple Ca^{2+} -releasing messengers. *Diabetes*. 2002;51 Suppl 3:349-57.

Carter CR, McKay CJ, Imrie CW. Percutaneous necrosectomy and sinus tract endoscopy in the management of infected pancreatic necrosis: an initial experience. *Ann Surg*. 2000;232(2):175-80

Case, RM & Argent BE. Pancreatic duct cell secretion: control and mechanisms of transport. In: *The Pancreas: Biology, Pathobiology and Disease*, eds. Go, VLW, Dimango EP, Gardner, J., Lebenthal, E., Reber, HA., & Scheele, GA. (1993) 301-350. Raven Press New York.

Chen CC, Wang SS, Tsay SH, Lee FY, Lu RH, Chang FY, Lee SD. Effects of nitric oxide synthase inhibitors on retrograde bile salt-induced pancreatitis rats. *J Chin Med Assoc*. 2004;67(1):9-14.

Cheng S, Zhao J, He SG, Song MM, Li ZH, Zhang YW. The role of nitric oxide in lung injury associated with acute necrotizing pancreatitis. *Zhonghua Wai Ke Za Zhi*. 2003;41(5):336-9.

Chey, WY., VLW, Dimango EP, Gardner, J., Lebenthal, E., Reber, HA., Scheele, GA. Hormonal control of pancreatic exocrine secretion. In: The Pancreas: Biology, Pathobiology and Disease, (1993) pp 403-424. Raven Press New York.

Chvanov M, Gerasimenko OV, Petersen OH, Tepikin AV. Calcium-dependent release of NO from intracellular S-nitrosothiols. *EMBO J.* 2006;25(13):3024-32

Connor S, Raraty MG, Howes N, Evans J, Ghaneh P, Sutton R, Neoptolemos JP. Surgery in the treatment of acute pancreatitis--minimal access pancreatic necrosectomy. *Scand J Surg.* 2005;94(2):135-42

Connor S, Ghaneh P, Raraty M, Rosso E, Hartley MN, Garvey C, Hughes M, McWilliams R, Evans J, Rowlands P, Sutton R, Neoptolemos JP. Increasing age and APACHE II scores are the main determinants of outcome from pancreatic necrosectomy. *Br J Surg.* 2003;90(12):1542-8

Connor S, Neoptolemos JP. Surgery for pancreatic necrosis: "whom, when and what". *World J Gastroenterol.* 2004;15;10(12):1697-8

Cosker F, Cheviron N, Yamasaki M, Menteyne A, Lund FE, Moutin MJ, Galione A, Cancela JM. The ecto-enzyme CD38 is a nicotinic acid adenine dinucleotide phosphate (NAADP) synthase that couples receptor activation to Ca²⁺ mobilization from lysosomes in pancreatic acinar cells. *J Biol Chem.* 2010;3;285(49):38251-9.

Criddle DN, Raraty MG, Neoptolemos JP, Tepikin AV, Petersen OH, Sutton R. Ethanol toxicity in pancreatic acinar cells: mediation by nonoxidative fatty acid metabolites. *Proc Natl Acad Sci U S A.* 2004;101(29):10738-43

Criddle DN, Murphy JA, Fistetto G, Barrow S, Tepikin AV, Neoptolemos JP, Sutton R, Petersen OH. Fatty acid ethyl esters cause pancreatic calcium toxicity via inositol trisphosphate receptors and loss of ATP synthesis. *Gastroenterology* 2006;130(3):781-93

Criddle DN, Sutton R, Petersen OH. Role of Ca²⁺ in pancreatic cell death induced by alcohol metabolites. *J Gastroenterol Hepatol.* 2006 ;21

Criddle DN, McLaughlin E, Murphy JA, Petersen OH, Sutton R. The pancreas misled: signals to pancreatitis. *Pancreatology.* 2007;7(5-6):436-46

Criddle DN, Gerasimenko JV, Baumgartner HK, Jaffar M, Voronina S, Sutton R, Petersen OH, Gerasimenko OV. Calcium signalling and pancreatic cell death: apoptosis or necrosis? *Cell Death Differ.* 2007;14(7):1285-94

Daniel PM, Henderson JR. The effect of vagal stimulation on plasma insulin and glucose levels in the baboon. *J Physiol.* 1967;192(2):317-27

Deng X, Wang L, Elm MS, Gabazadeh D, Diorio GJ, Eagon PK, Whitcomb DC. Chronic alcohol consumption accelerates fibrosis in response to cerulein-induced pancreatitis in rats. *Am J Pathol* 2005;166:93-106.

Dickson AP, O'Neill J, Imrie CW. Hyperlipidaemia, alcohol abuse and acute pancreatitis. *Br J Surg* 1984;71:685-8.

Diczfalusy MA, Bjorkhem I, Einarsson C, Hillebrant CG, Alexson SE. Characterization of enzymes involved in formation of ethyl esters of long-chain fatty acids in humans. *J Lipid Res* 2001;42:1025-32.

DiMagno MJ, Williams JA, Hao Y, Ernst SA, Owyang C. Endothelial nitric oxide synthase is protective in the initiation of caerulein-induced acute pancreatitis in mice. *Am J Physiol Gastrointest Liver Physiol.* 2004;287(1):G80-7.

Dolman NJ, Tepikin AV. Calcium gradients and the Golgi. *Cell Calcium.* 2006; 40(5-6):505-12

Douglas WW, Rubin RP. The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *Jour of Physiol* 1961; 159:40-57

Douglas WW. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br Jour of Pharm* 1968;34:451-474

Dufresne M, Seva C, Fourmy D. Cholecystokinin and gastrin receptors. *Physiol Rev* 2006;86:805-847.

Durbec JP, Sarles H. Multicenter survey of the etiology of pancreatic diseases. Relationship between the relative risk of developing chronic pancreatitis and alcohol, protein and lipid consumption. *Digestion* 1978;18:337-50.

Echenique AM, Sleeman D, Yrizarry J, Scagnelli T, Guerra JJ Jr, Casillas VJ, Huson H, Russell E. Percutaneous catheter-directed debridement of infected pancreatic necrosis: results in 20 patients. *J Vasc Interv Radiol.* 1998;9(4):565-71

Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, Brand MD. Superoxide activates mitochondrial uncoupling proteins. *Nature* 2002;415:96-9.

Ehrlich BE, Kaftan E, Bezprozvannaya S, Bezprozvanny I. The pharmacology of intracellular Ca^{2+} -release channels. *Trends Pharmacol Sci* 1994;15:145-9.

Eysselein VE, Eberlein GA, Schaeffer M, Grandt D, Goebell H, Niebel W, Rosenquist GL, Meyer HE, Reeve JR Jr. Characterization of the major form of cholecystokinin in human intestine: CCK-58. *Am J Physiol* 1990;258:G253-60.

Eysselein VE, Eberlein GA, Hesse WH, Schaeffer M, Grandt D, Williams R, Goebell H, Reeve JR Jr. Molecular variants of cholecystokinin after endogenous stimulation in humans: a time study. *Am J Physiol* 1990;258:G951-957.

Fernandez-del Castillo C, Rattner DW, Makary MA, Mostafavi A, McGrath D, Warshaw AL. Debridement and closed packing for the treatment of necrotizing pancreatitis. *Ann Surg.* 1998;228(5):676-84.

Folsch UR, Nitsche R, Ludtke R, et al. Early ERCP and papillotomy compared with conservative treatment for acute biliary pancreatitis: the German Study Group on Acute Biliary Pancreatitis. *N Engl J Med.* 1997;336:237-242.

Fortunato F, Deng X, Gates LK, McClain CJ, Bimmler D, Graf R, Whitcomb DC. Pancreatic response to endotoxin after chronic alcohol exposure: switch from apoptosis to necrosis? *Am J Physiol Gastrointest Liver Physiol.* 2006;290(2):G232-41

Freeny PC, Hauptmann E, Althaus SJ, Traverso LW, Sinanan M. Percutaneous CT-guided catheter drainage of infected acute necrotizing pancreatitis: techniques and results. *AJR Am J Roentgenol.* 1998;170(4):969-75

Friberg H and Wieloch T. Mitochondrial permeability transition in acute neurodegeneration, *Biochimie*, 2002. Volume 84, Issues 2-3, Pages 241-250.

Frick TW, Hailemariam S, Heitz PU, Largiader F, Goodale RL. Acute hypercalcemia induces acinar cell necrosis and intraductal protein precipitates in the pancreas of cats and guinea pigs. *Gastroenterology.* 1990;98:1675-81

Frick T, Spycher M, Kaiser A, Goodale RL, Largiader F. Electron microscopy of the exocrine pancreas in experimental acute hypercalcemia *Helv Chir Acta.* 1991; 57(5):713-6

Frick TW, Spycher MA, Heitz PU, Largiader F, Goodale RL. Hypercalcaemia and pancreatic ultrastructure in cats. *Eur J Surg.* 1992; 158(5):289-94

Frick TW, Wiegand D, Bimmler D, Fernandez-del Castillo C, Rattner DW, Warshaw AL. A rat model to study hypercalcemia-induced acute pancreatitis. *Int J Pancreatol.* 1994; 15(2):91-6.

Frick TW, Mithofer K, Fernandez-del Castillo C, Rattner DW, Warshaw AL. Hypercalcemia causes acute pancreatitis by pancreatic secretory block, intracellular zymogen accumulation, and acinar cell injury. *Am J Surg.* 1995;169(1):167-72.

Fugger R, Schulz F, Rogy M, Herbst F, Mirza D, Fritsch A. Open approach in pancreatic and infected pancreatic necrosis: laparostomies and preplanned revisions. *World J Surg.* 1991;15(4):516-20

Futatsugi A, Nakamura T, Yamada MK, Ebisui E, Nakamura K, Uchida K, Kitaguchi T, Takahashi-Iwanaga H, Noda T, Aruga J, Mikoshiba K. IP3 receptor types 2 and 3 mediate exocrine secretion underlying energy metabolism. *Science* 2005;309:2231-2234.

Galindo J, Jones N, Powell GL, Hollingsworth SJ, Shankley N. Advanced qRT-PCR technology allows detection of the cholecystokinin 1 receptor (CCK1R) expression in human pancreas. *Pancreas* 2005;31:325-331.

Gallione A, Petersen OH. The NAADP Receptor: New Receptors or New Regulation? *Mol Interv.* 2005 ;5(2):73-9.

Gerasimenko OV, Gerasimenko JV, Belan PV, Peterson OH. Inositol triphosphate and cyclic ADP-ribose mediated release of calcium from isolated pancreatic zymogen granules. *Cell* 1996; 84: 473 -80

Gerasimenko JV, Tepikin AV, Peterson OHN, Gerasimenko OV. Calcium uptake via endocytosis with rapid release from acidifying endosomes. *Curr Biol* 1998; 8: 1335-8

Gerasimenko JV, Gerasimenko OV, Palejwala A, Tepikin AV, Peterson OH, Watson AJ. Menindione –induced apoptosis: roles of cytosolic calcium elevations and the mitochondrial permeability transition pore. *J Cell Sci* 2002; 115: 485-97

Gerasimenko JV, Flowerdew SE, Voronina SG, Sukhomlin TK, Tepikin AV, Petersen OH, Gerasimenko OV. Bile acids induce Ca^{2+} release from both the endoplasmic reticulum and acidic intracellular calcium stores through activation of inositol trisphosphate receptors and ryanodine receptors. *J Biol Chem.* 2006 29;281(52):40154-63.

Gijs J. D. van Acker, Ashok K. Saluja, Lakshmi Bhagat, Vijay P. Singh, Albert M. Song, and Michael L. Steer. Cathepsin B inhibition prevents trypsinogen activation and reduces pancreatitis severity *Am J Physiol Gastrointest Liver Physiol* Vol. 283, Issue 3, G794-G800

Go VL, Gukovskaya A, Pandol SJ. Alcohol and pancreatic cancer. *Alcohol.* 2005;35(3):205-11.

Goebell H. The role of calcium in pancreatic secretion and disease. *Acta Hepatogastroenterol (Stuttg)*. 1976;23(2):151-61

Gorelick F.S. and Jamieson J.D. The pancreatic acinar cell: structure – function relationship. In: *The Physiology of the Gastrointestinal Tract*, ed. Johnson, L. (1994). pp 1353-1376. Raven Press, New York.

Gorelick FS, Matovcik LM. Lysosomal enzymes and pancreatitis. *Gastroenterology* 1995; 109: 620-5

Grundy SM. Hypertriglyceridemia, atherogenic dyslipidemia, and the metabolic syndrome. *Am J Cardiol* 1998;81:18B-25B.

Guicciardi ME, Deussing J, Miyoshi H, Bronk SF, Svingen PA, Peters C, Kaufmann SH, Gores GJ. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. *J Clin Invest*. 2000;106(9):1127-37.

Guicciardi ME, Miyoshi H, Bronk SF, Gores GJ. Cathepsin B knockout mice are resistant to tumor necrosis factor-alpha-mediated hepatocyte apoptosis and liver injury: implications for therapeutic applications. *Am J Pathol*. 2001;159(6):2045-54.

Gukovskaya AS, Mouria M, Gukovsky I, Reyes CN, Kasho VN, Faller LD, Pandol SJ. Ethanol metabolism and transcription factor activation in pancreatic acinar cells in rats. *Gastroenterology* 2002;122:106-18.

Gukovskaya AS, Hosseini S, Satoh A, Cheng JH, Nam KJ, Gukovsky I, Pandol SJ. Ethanol differentially regulates NF-kappaB activation in pancreatic acinar cells through calcium and protein kinase C pathways. *Am J Physiol Gastrointest Liver Physiol*. 2004;286(2):G204-13

Gukovsky I, Cheng JH, Nam KJ, Lee OT, Lugea A, Fischer L, Penninger JM, Pandol SJ, Gukovskaya AS. Phosphatidylinositide 3-kinase gamma regulates key pathologic responses to cholecystokinin in pancreatic acinar cells. *Gastroenterology* 2004;126:554-66.

Hajnoczky G, Robb-Gaspers LD, Seitz MB, Thomas AP. Decoding of cytosolic calcium oscillations in the mitochondria. *Cell* 1995;82:415-424.

Halangk W, Matthias R, Nedelew B, Schild L, Meyer F, Schulz HU, Lippert H. Modification of energy supply by pancreatic mitochondria in acute experimental pancreatitis. *Zentralbl Chir*. 1997;122(4):305-8

Halangk W, Lerch MM, Brandt-Nedelev B, Roth W, Ruthenbuerger M, Reinheckel T, Domschke W, Lippert H, Peters C, Deussing J. Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. *J Clin Invest* 2000; 106: 773-81

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 1981;391(2):85-100

Han B, Logsdon CD. CCK stimulates mob-1 expression and NF-kappaB activation via protein kinase C and intracellular calcium. *Am J Physiol Cell Physiol* 2000; 278: C344-51

Haworth RA and Hunter DR. 1979. The Ca²⁺-induced membrane transition in mitochondria II. Nature of the Ca²⁺ trigger site. *Archives of Biochemistry and Biophysics*, Volume 195, Issue 2, Pages 460-467.

Hegy P, Rakonczay Z Jr, Tiszlavicz L, Varró A, Tóth A, Rácz G, Varga G, Gray MA, Argent BE. SLC26 transporters and the inhibitory control of pancreatic ductal bicarbonate secretion. *Novartis Found Symp.* 2006;273:164-73

Hegy P, Rakonczay Z Jr. The inhibitory pathways of pancreatic ductal bicarbonate secretion. *Int J Biochem Cell Biol.* 2007;39(1):25-30. 2006

Henderson JR. Why are the islets of Langerhans? *Lancet* (1969)2, 469 – 470

Henze K, Martin W. "Evolutionary biology: essence of mitochondria". *Nature* 2003,426 (6963): 127–8.

Herrmann JM, Neupert W. Protein transport into mitochondria. *Curr Opin Microbiol* 2000. 3 (2): 210–214

Hietaranta AJ, Singh VP, Bhagat L, van Acker GJ, Song AM, Mykoniatis A, Steer ML, Saluja AK. Water immersion stress prevents caerulein induced pancreatic acinar cell NF-kappaB activation by attenuating caerulein induced intracellular calcium changes. *J Biol Chem* 2001; 276: 18742-7

Hietaranta AJ, Saluja AK, Bhagat L, Singh VP, Song AM, Steer ML. Relationship between NF kappaB and trypsinogen activation in rat pancreas after supramaximal caerulein stimulation. *Biochem Biophys Res Comm* 2001; 280: 388-95

Hofbauer B, Friess H, Weber A, Baczako K, Kisling P, Schilling M, Uhl W, Dervenis C, Buchler MW. Hyperlipaemia intensifies the course of acute oedematous and acute necrotising pancreatitis in the rat. *Gut* 1996;38:753-8.

Hofbauer B, Saluja AK, Lerch MM, Bhagat L, Bhatia M, Lee HS, Frossard JL, Alder G, Steer ML, Intra-acinar cell activation of trypsinogen during caerulein induced pancreatitis in rats. *Ann J Physiol Gastrointest Liver Physiol* 1998;275; G352-62

Holst, JJ. Neural regulation of pancreatic exocrine function. In *The Pancreas: Biology, Pathobiology and Disease*, eds. Go, VLW., Dimango EP, Gardner, J., Lebenthal, E., Reber HA., & Scheele, GA., (1993). pp. 381-402. Raven Press, New York.

Howes N, Neoptolemos JP. Risk of pancreatic ductal adenocarcinoma in chronic pancreatitis. *Gut*. 2002;51(6):765-6

Howes N, Greenhalf W, Stocken DD, Neoptolemos JP. Cationic trypsinogen mutations and pancreatitis. *Clin Lab Med*. 2005;25(1):39-59.

Hunt MC, Nousiainen SE, Huttunen MK, Orii KE, Svensson LT, Alexson SE. Peroxisome proliferator-induced long chain acyl-CoA thioesterases comprise a highly conserved novel multi-gene family involved in lipid metabolism. *J Biol Chem* 1999;274:34317-26

Husain SZ, Prasad P, Grant WM, Kolodecik TR, Nathanson MH, Gorelick FS. The ryanodine receptor mediates early zymogen activation in pancreatitis. *Proc Natl Acad Sci U S A* 2005;102:14386-14391.

Ichlas F and Mazat JP. 1998. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. *Biochimica et Biophysica Acta*, Volume 1366, Issues 1-2, Pages 33-50

Imrie CW, McKay CJ. The scientific basis of medical therapy of acute pancreatitis. Could it work, and is there a role for lexipafant? *Gastroenterol Clin North Am*. 1999;28(3):591-9

Inoue M, Fujishiro N, Imanaga I, Sakamoto Y. Role of ATP decrease in secretion induced by mitochondrial dysfunction in guinea-pig adrenal chromaffin cells. *J Physiol* 2002;539:145-155.

Isemann R, Runzi M, Kron M, Kahl S, Kraus D, Jung N, Maier L, Malfertheiner P, Goebell H, Beger HG. Prophylactic antibiotic treatment in patients with predicted severe acute pancreatitis: a placebo-controlled, double-blind trial. *Gastroenterology*. 2004;126(4):997-1004.

Jaburek M, Varecha M, Gimeno RE, Dembski M, Jezek P, Zhang M, Burn P, Tartaglia LA, Garlid KD. Transport function and regulation of mitochondrial uncoupling proteins 2 and 3. *J Biol Chem* 1999;274:26003-7.

Jensen RT, Wank SA, Rowley WH, Sato S, Gardner JD. Interaction of CCK with pancreatic acinar cells. *Trends Pharmacol Sci* 1989;10:418-423.

Jensen RT. Involvement of cholecystokinin/gastrin-related peptides and their receptors in clinical gastrointestinal disorders. *Pharmacol Toxicol* 2002;91:333-50.

Ji B, Bi Y, Simeone D, Mortensen RM, Logsdon CD. Human pancreatic acinar cells lack functional responses to cholecystokinin and gastrin. *Gastroenterology*. 2001;121(6):1380-90.

Ji B, Bi Y, Simeone D, Mortensen RM, Logsdon CD. Human pancreatic acinar cells do not respond to cholecystokinin. *Pharmacol Toxicol*. 2002 91(6):327-32.

Johnson CD, Kingsnorth AN, Imrie CW, McMahon MJ, Neoptolemos JP, McKay C, Toh SK, Skaife P, Leeder PC, Wilson P, Larvin M, Curtis LD. Double blind, randomised, placebo controlled study of a platelet activating factor antagonist, lexipafant, in the treatment and prevention of organ failure in predicted severe acute pancreatitis. *Gut*. 2001;48(1):62-9.

Kamisawa T, Matsukawa M, Amemiya K, Tu Y, Egawa N, Okamoto A, Aizawa S. Pancreatitis associated with pancreaticobiliary maljunction. *Hepatogastroenterology*. 2003;50(53):1665-8

Karimngani I, Porter KA, Langevin RE, Banks PA. Prognostic factors in sterile pancreatic necrosis. *Gastroenterology*. 1992;103(5):1636-40

Katz M, Carangelo R, Miller LJ, Gorelick F. Effect of ethanol on cholecystokinin-stimulated zymogen conversion in pancreatic acinar cells. *Am J Physiol* 1996;270:G171-5.

Kern, H.F. (1993) Fine structure of the human exocrine pancreas. In: *The Pancreas: Biology, Pathobiology and Disease*, Go, V.L.W., Dimango, W.P., Gardner, J., Lebenthal, E., Reber, H.A., & Scheel, G.A., pp.3. 2nd ed. Raven Press, New York.

Kim JY, Kim KH, Lee JA, Namkung W, Sun AQ, Ananthanarayanan M, Suchy FJ, Shin DM, Muallem S, Lee MG. Transporter-mediated bile acid uptake causes Ca^{2+} -dependent cell death in rat pancreatic acinar cells. *Gastroenterology*. 2002; 122(7):1941-53.

King A, Selak MA, Gottlieb E. "Succinate dehydrogenase and fumarate hydratase: linking mitochondrial dysfunction and cancer". *Oncogene*. (2006)25 (34): 4675–4682.

Klonowski-Stumpe H, Luthen R, Han B, Sata N, Haussinger D, Niederau C Inhibition of cathepsin B does not affect the intracellular activation of trypsinogen by cerulein hyperstimulation in isolated rat pancreatic acinar cells. *Pancreas*. 1998;16(1):96-101

Klonowski-Stumpe H, Schreiber R, Grolik M, Schulz HU, Haussinger D, Niederau C. Effect of oxidative stress on cellular functions and cytosolic free calcium of rat pancreatic acinar cells. *Am J Physiol.* 1997;272, G1489-98

Kono H, Nakagami M, Rusyn I, Connor HD, Stefanovic B, Brenner DA, Mason RP, Arteel GE, Thurman RG. Development of an animal model of chronic alcohol-induced pancreatitis in the rat. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G1178-86.

Kreis ME, Zittel TT, Raybould HE, Reeve JR, Grundy D. Prolonged intestinal afferent nerve discharge in response to cholecystokinin-58 compared to cholecystokinin-8 in rats. *Neurosci Lett* 1997;230:89-92.

Kruger B, Albrecht E, Lerch MM The role of intracellular calcium signalling in premature protease activation and the onset of pancreatitis. *Am J Pathol.* 2000; 157(1):43-50.

Kubisch CH, Gukovsky I, Lugea A, Pandol SJ, Kuick R, Misek DE, Hanash SM, Logsdon CD. Long-term ethanol consumption alters pancreatic gene expression in rats: a possible connection to pancreatic injury. *Pancreas.* 2006;33(1):68-76.

Lange LG. Nonoxidative ethanol metabolism: formation of fatty acid ethyl esters by cholesterol esterase. *Proc Natl Acad Sci U S A.* 1982;79(13):3954-7.

Lange LG, Sobel BE. Mitochondrial dysfunction induced by fatty acid ethyl esters, myocardial metabolites of ethanol. *J Clin Invest* 1983;72:724-31.

Lange E, Schult H, Lorenz W, Reimann HJ, Maroske D, Neuhaus H, Schwarz B, Kresse U. Histamine and pancreatitis: increase of plasma histamine levels in dogs with Pfeffer preparation and influence of aminoguanidine on the survival time. *Agents Actions*. 1978 ;8(4):376-9.

Laposata EA, Lange LG. Presence of nonoxidative ethanol metabolism in human organs commonly damaged by ethanol abuse. *Science* 1986;231:497-9.

Lau BW, Colella M, Ruder WC, Ranieri M, Curci S, Hofer AM. Deoxycholic acid activates protein kinase C and phospholipase C via increased Ca^{2+} entry at plasma membrane. *Gastroenterology* 2005;128:695-707.

Lebenthal E. Hormonal control of pancreatic exocrine secretion. In: *The Pancreas: Biology, Pathobiology and Disease*, eds. Go, VLW, Dimango EP, Gardner, J.Reber, HA., & Scheele, GA., (1993). pp 1041- 1081. Raven Press New York.

Leach SD, Modlin IM, Scheele GA, Gorelick FS. Intracellular activation of digestive zymogens in rat pancreatic acini. stimulated by high doses of cholecystokinin. *J Clin Invest* 1991; 87; 362-6

Lee MG, Xu X, Zeng W, Diaz J, Wojcikiewicz RJ, Kuo TH, Wuytack F, Racymaekers L, Muallem S. Polarized expression of Ca²⁺ channels in pancreatic and salivary gland cells. Correlation with initiation and propagation of [Ca²⁺]_i waves. *J Biol Chem* 1997;272:15765-70.

Lee MJ, Rattner DW, Legemate DA, Saini S, Dawson SL, Hahn PF, Warshaw AL, Mueller PR. Acute complicated pancreatitis: redefining the role of interventional radiology. *Radiology*. 1992;183(1):171-4

Leindler L, Morschl E, Laszlo F, Mandi Y, Takacs T, Jarmai K, Farkas G. Importance of cytokines, nitric oxide, and apoptosis in the pathological process of necrotizing pancreatitis in rats. *Pancreas*. 2004;29(2):157-61.

Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, and Herman B. 1998. The mitochondrial permeability transition in cell death: A common mechanism in necrosis, apoptosis and autophagy. *Biochimica et Biophysica Acta*. Volume 1366, Issues 1–2, Pages 177–196

Lerch MM, Saluja AK, Runzi M, Dawra R, Saluja M, Steer ML. Pancreatic duct obstruction triggers acute necrotizing pancreatitis in the opossum. *Gastroenterology*. 1993;104(3):853-61

Lerch MM, Saluja AK, Dawra R, Ramarao P, Saluja M, Steer ML. Acute necrotizing pancreatitis in the opossum: earliest morphological changes involve acinar cells. *Gastroenterology* 1992;103(1):205-13.

Letko G, Siech M, Sokolowski A, Spormann H. Experimental acute pancreatitis in rats after chronic and chronic plus acute ethanol administration in combination with a pancreatic juice oedema. *Int Surg.* 1989;74(2):77-80.

Leyssens A, Nowicky AV, Patterson L, Crompton M, Duchon MR. The relationship between mitochondrial state, ATP hydrolysis, $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$ studied in isolated rat cardiomyocytes. *J Physiol* 1996;496:111-128.

Li Y, Owyang C. Vagal afferent pathway mediates physiological action of cholecystokinin on pancreatic enzyme secretion. *J Clin Invest* 1993;92:418-424.

Liddle RA. Cholecystokinin cells. *Annu Rev Physiol* 1997;59:221-42

Lifson N, Kramlinger KG, Mayrand RR, Lender EJ. Blood flow to the rabbit pancreas with special reference to the islets of Langerhans. *Gastroenterology.* 1980;79(3):466-73

Lifson N, Lassa CV, Dixit PK. Relation between blood flow and morphology in islet organ of rat pancreas. *Am J Physiol.* 1985 ;249(1 Pt 1):E43-8.

Lombardi B, Estes LW, Longnecker DS. Acute hemorrhagic pancreatitis (massive necrosis) with fat necrosis induced in mice by DL-ethionine fed with a choline-deficient diet. *Am J Pathol.* 1975;79(3):465-80.

Lu Z, Karne S, Kolodecik T. Alcohols enhance caerulein induced zymogen activation in pancreatic acinar cells. *Am J Physiol Gastrointest Liver Physiol* 2002; 282: G501 – G507

Makela A, Kuusi T, Schroder T. Inhibition of serum phospholipase-A2 in acute pancreatitis by pharmacological agents in vitro. *Scand J Clin Lab Invest.* 1997;57(5):401-7.

Makhija R, Kingsnorth A. Cytokine storm in acute pancreatitis. *J Hepatobiliary Pancreat Surg* 2002; 9: 401-410

Maruyama Y, Inooka G, Li YX, Miyashita Y, Kasai H. Agonist-induced localized Ca²⁺ spikes directly triggering exocytotic secretion in exocrine pancreas. *EMBO J* 1993; 12: 3017 – 3022

Matozaki T, Goke B, Tsunoda Y, Rodriguez M, Williams JA. Two functionally distinct cholecystokinin receptors show different modes of action on calcium mobilisation and phospholipids hydrolysis in isolated rat pancreatic acini. Studies using a new cholecystokinin analogue JMV -180. *J Biol Chem* 1990; 265: 6247 – 54

Mayerle J, Schneckeburger J, Krüger B, Kellermann J, Ruthenbürger M, Weiss FU, Nalli A, Domschke W, Lerch MM. Extracellular cleavage of E-cadherin by leukocyte elastase during acute experimental pancreatitis in rats. *Gastroenterology*. 2005;129(4):1251-67

McBride HM, Neuspiel M, Wasiak. Mitochondria: more than just a powerhouse. *Curr. Biol. S* (2006)16 (14): R551.

McKay CJ, Evans S, Sinclair M, Carter CR, Imrie CW. High early mortality rate from acute pancreatitis in Scotland, 1984-1995. *Br J Surg*. 1999;86(10):1302-5.

McKay CJ, Imrie CW. The continuing challenge of early mortality in acute pancreatitis. *Br J Surg*. 2004;91(10):

Menger MD, Plusczyk T, Vollmar B. Microcirculatory derangements in acute pancreatitis. *J Hepatobiliary Pancreat Surg*. 2001;8(3):187-94.

Miller LJ. Does the human pancreas have a type A or B personality? *Gastroenterology* 1996;111: 1767-1770.

Mithofer K, Fernandez-del Castillo C, Frick TW, Foitzik T, Bassi DG, Lewandrowski KB, Rattner DW, Warshaw AL. Increased intrapancreatic trypsinogen activation in ischemia-induced experimental pancreatitis. *Ann Surg.* 1995; 221(4): 364-71.

Mithofer K, Fernandez – del Castillo C, Rattner D, Warshaw AL. Subcellular kinetics of early trypsinogen activation in acute rodent pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 1998; 274; G71-9

Mogami H, Nakano K, Tepikin AV, Peterson OH. Calcium flow via tunnels in polarised cells: recharging of apical calcium stores by focal calcium entry through basal membrane patch. *Cell* 1997; 88: 49-55

Mogami H, Tepikin AV, Peterson OH. Termination of cytosolic calcium signals. Calcium reuptake into intracellular stores is regulated by the free calcium concentration in the store lumen. *EMBO J* 1998; 17: 435-42

Mogami H, Gardner J, Gerasimenko OV, Camello P, Peterson OH, Tepikin AV. Calcium binding capacity of the cytosol and endoplasmic reticulum of mouse pancreatic acinar cells. *J Physiol* 1999; 518: 463-467

Moore and Persaud. *Before we are born – Essentials of embryology and birth defects*, 4th Edition, 193-194. W.B. Saunders Company.

Mooren FCh, Hlouschek V, Finkes T, Turi S, Weber IA, Singh J, Domschke W, Schnekenburger J, Kruger B, Lerch MM. Early changes in pancreatic acinar cell calcium signaling after pancreatic duct obstruction. *J Biol Chem* 2003;278:9361-9.

Morton C, Klatsky AL, Udaltsova N. Smoking, coffee, and pancreatitis. *Am J Gastroenterol.* 2004 ;99(4):731-8

Mukherjee R, Criddle DN, Gukvoskaya A, Pandol S, Petersen OH, Sutton R. Mitochondrial injury in pancreatitis. *Cell Calcium.* 2008

Murray B, Carter R, Imrie C, Evans S, O'Suilleabhain C. Diclofenac reduces the incidence of acute pancreatitis after endoscopic retrograde cholangiopancreatography. *Gastroenterology.* 2003;124(7):1786-91.

Muruyama Y, Inooka G, Li YX, Miyashita Y, Kasai H. Agonist-induced localized Ca^{2+} spikes directly triggering exocytotic secretion in exocrine pancreas. *EMBO J* 1993;12:3017-3022.

Nathanson MH, Fallon MB, Padfield PJ, Maranto AR. Localization of the type-3 inositol-1,4,5-trisphosphate receptor in the Ca^{2+} wave trigger zone of pancreatic acinar cells. *J Biol Chem* 1994;269:4693-4696

Neblett WW 3rd, O'Neill JA Jr. Surgical management of recurrent pancreatitis in children with pancreas divisum. *Ann Surg.* 2000;231(6):899-908

Niederau C, Schulz HU. Current conservative treatment of acute pancreatitis: evidence from animal and human studies. *Hepatogastroenterology.* 1993;40(6):538-49.

Niederau C, Niederau M, Luthen R, Strohmeyer G, Ferrell LD, Grendell JH. Pancreatic exocrine secretion in acute experimental pancreatitis. *Gastroenterology* 1990;99:1120-7

Neoptolemos JP, Carr-Locke DL, London NJ, et al. Controlled trial of urgent endoscopic retrograde cholangiopancreatography and endoscopic sphincterotomy versus conservative treatment for acute pancreatitis due to gallstones. *Lancet.* 1988;2:979-983

Neoptolemos JP, Raraty M, Finch M, Sutton R. Acute pancreatitis: the substantial human and financial costs. *Gut* 1998; 42; 886 -91

Nordback IH, Clemens JA, Chacko VP, Olson JL, Cameron JL. Changes in high-energy phosphate metabolism and cell morphology in four models of acute experimental pancreatitis. *Ann Surg* 1991;213:341-9.

Nguyen, T., Chin, W. C., and Verdugo, P. (1998) *Nature* 395, 908-912

O'Konski MS, Pandol SJ. Effects of caerulein on the apical cytoskeleton of the pancreatic acinar cell. *J Clin Invest* 1990; 86; 1649-57

Opie E.L. The aetiology of acute haemorrhagic pancreatitis. *John Hopkins Hospital Bulletin* 1901; 12; 182-188

Osipchuk YV, Wakui M, Yule DI, Gallacher DV, Petersen OH. Cytoplasmic Ca^{2+} oscillations evoked by receptor stimulation, G-protein activation, internal application of inositol trisphosphate or Ca^{2+} : simultaneous microfluorimetry and Ca^{2+} dependent Cl^- current recording in single pancreatic acinar cells. *EMBO J* 1990;9:697-704.

Owyang C, Logsdon CD. New insights into neurohormonal regulation of pancreatic secretion. *Gastroenterology*. 2004;127(3):957-69.

Pahl HL. Signal transduction from the endoplasmic reticulum to the cell nucleus. *Physiol Rev* 1999; 79: 683-701

Palade G. Intracellular aspects of the process of protein synthesis. *Science* 1975; 189: 347-358

Pandol SJ, Periskic S, Gukovsky I, Zaninovic V, Jung Y, Zong Y, Solomon TE, Gukovskaya AS, Tsukamoto H. Ethanol diet increases the sensitivity of rats to pancreatitis induced by cholecystokinin octapeptide. *Gastroenterology* 1999;117:706-16.

Pandol, SJ, Gukovsky I, Satoh A, Lugea A, Gukovskaya AS. Animal and in vitro models of alcoholic pancreatitis: role of cholecystokinin. *Pancreas* 2003;27:297-300

Pandol SJ. Are we studying the correct state of the stellate cell to elucidate mechanisms of chronic pancreatitis? *Gut*. 2005;54(6):744-5

Pandol SJ, Saluja AK, Imrie CW, Banks PA. Acute pancreatitis: bench to the bedside *Gastroenterology*. 2007;132(3):1127-51.

Park MK, Petersen OH, Tepikin AV. The endoplasmic reticulum as one continuous calcium pool: visualisation of rapid calcium movements and equilibration. *EMBO J* 2000; 19: 5729-5739

Paschen W. Dependence of vital cell function on endoplasmic reticulum levels: implications for the mechanisms underlying neuronal cell injury in different pathological states. *Cell Calcium* 2001; 29: 1-11

Park MK, Ashby MC, Erdemli G, Peterson OH, Tepikin AV. Perinuclear, perigranular and sub-plasmalemmal mitochondria have distinct functions in the regulation of cellular calcium transport. *EMBO J* 2001; 20: 1863-74

Park M, Lomax RB, Tepikin AV, Peterson OH. Local uncaging of caged Ca^{2+} reveals distribution of Ca^{2+} -activated Cl^{-} channels in pancreatic acinar cells. *Proc Natl Acad Sci USA* 2001; 98:10948 – 10953

Park MK, Lee M, Petersen OH. Morphological and functional changes of dissociated single pancreatic acinar cells: testing the suitability of the single cell as a model for exocytosis and calcium signaling. *Cell Calcium*. 2004;35(4):367-79.

Pearson GT, Davison JS, Collins RC, Petersen OH. Control of enzyme secretion by non-cholinergic, non-adrenergic nerves in guinea pig pancreas. *Nature* 1981; 290:259-261.

Pearson GT, Singh J, Daoud MS, Davison JS, Petersen OH. Control of pancreatic cyclic nucleotide levels and amylase secretion by non-cholinergic, nonadrenergic nerves – a study employing electrical field stimulation of guinea-pig segments. *J Biol Chem* 1981;256:1025-1031.

Penzo D, Tagliapietra C, Colonna R, Petronilli V, Bernardi P. Effects of fatty acids on mitochondria: implications for cell death. *Biochim Biophys Acta* 2002;1555:160-5.

Perides G, A.Sharma, A.Gopal, X.Tao, K.Dwyer, B.Ligon, M.Steer. Secretin Differentially Sensitizes Rat Pancreatic Acini To The Effects Of Supramaximal Stimulation With Caerulin. *Am Journ Physiol* 2005 289(4):G713-21

Petersen OH, Findlay I, Iwatsuki N, Singh J, Gallacher DV, Fuller CM, Pearson GT, Dunne MJ, Morris AP. Human pancreatic acinar cells: studies of stimulus-secretion coupling. *Gastroenterology* 1985;89:109-117.

Petersen OH. Stimulus-secretion coupling: cytoplasmic calcium signals and the control of ion channels in exocrine acinar cells. *J Physiol* 1992;448:1-51.

Petersen OH. Local and global Ca^{2+} signals: physiology and pathophysiology. *Biol Res.* 2004;37(4): 661-4.

Petersen OH. Ca^{2+} signalling and Ca^{2+} -activated ion channels in exocrine acinar cells. *Cell Calcium.* 2005;38(3-4):171-200.

Petersen OH, Sutton R. Ca^{2+} signalling and pancreatitis: effects of alcohol, bile and coffee. *Trends Pharmacol Sci.* 2006;27(2):113-20

Petersen OH, Tepikin AV. Polarized calcium signalling in exocrine gland cells. *Annu Rev Physiol* 2008; 70:273-299.

Pfutzer RH, Tadic SD, Li HS, Thompson BS, Zhang JY, Ford ME, Eagon PK, Whitcomb DC. Pancreatic cholesterol esterase, ES-10, and fatty acid ethyl ester synthase III gene expression are increased in the pancreas and liver but not in the brain or heart with long-term ethanol feeding in rats. *Pancreas* 2002;25:101-6.

Ponappa BC, Ellingson JS, Hoek JB, Rubin E. Fatty acid ethyl ester formation resulting from ethanol exposure may increase the susceptibility of pancreas to ethanol – related tissue injury. *FASEB J* 1995; 9: A704

Ponappa BC, Marciniak R, Schneider T. Ethanol consumption and susceptibility of the pancreas to caerulein – induced pancreatitis. *Pancreas* 1997;14:150-157

Pralong WF, Bartley C, Wollheim CB. Single islet beta-cell stimulation by nutrients: relationship between pyridine nucleotides, cytosolic Ca^{2+} and secretion. *EMBO J* 1990;9:53-60.

Ramo O. Antecedent long term ethanol consumption in combination with different diets alters the severity of experimental acute pancreatitis in rats. *Gut* 1987;28:64-69

Raraty M, Peterson O.H, Sutton R, Neoptolemos J.P. Intracellular free ionised calcium in the pathogenesis of acute pancreatitis. *Ball Clin Gastro* 1999; 13: 2: 241-251

Raraty M, Ward J, Erdemli G, Vaillant C, Neoptolemos JP, Sutton R, Peterson OH. Calcium-dependent enzyme activation and vacuole formation in the apical granular region of pancreatic acinar cells. PNAS; 2000;97(24): 13126-13131

Raraty M, Neoptolemos JP, Peterson OH, Sutton R. Initiation, site and sequence of intracellular enzyme activation in early experimental acute pancreatitis. Pancreas 2002; 25: 446

Raraty MG, Connor S, Criddle DN, Sutton R, Neoptolemos JP. Acute pancreatitis and organ failure: pathophysiology, natural history, and management strategies. Curr Gastroenterol Rep. 2004;6(2):99-103.

Raraty MG, Murphy JA, Mcloughlin E, Smith D, Criddle D, Sutton R. Mechanisms of acinar cell injury in acute pancreatitis. Scand J Surg. 2005;94(2):89-96.

Rattner DW, Legermate DA, Lee MJ, Mueller PR, Warshaw AL. Early surgical debridement of symptomatic pancreatic necrosis is beneficial irrespective of infection. Am J Surg. 1992 ;163(1):105-9.

Rau B, Pralle U, Uhl W, Schoenberg MH, Beger HG. Management of sterile necrosis in instances of severe acute pancreatitis. Journal Am Coll Surg. 1995;181(4):279-88.

Reeves EP, Lu H, Jacobs HL, Messina CG, Bolsover S, Gabella G, Potma EO, Warley A, Roes J, Segal AW. Killing activity of neutrophils is mediated through activation of proteases by potassium influx. *Nature* 2002; 416: 291-7

Reeve JR Jr, McVey DC, Bunnett NW, Solomon TE, Keire DA, Ho FJ, Davis MT, Lee TD, Shively JE, Vigna SR. Differences in receptor binding and stability to enzymatic digestion between CCK-8 and CCK-58. *Pancreas* 2002;25:e50-5.

Reeve JR Jr, Green GM, Chew P, Eysselein VE, Keire DA. CCK-58 is the only detectable endocrine form of cholecystokinin in rat. *Am J Physiol Gastrointest Liver Physiol.* 2003;285(2):G255-65

Reeve JR Jr, Wu SV, Keire DA, Faull K, Chew P, Solomon TE, Green GM, Coskun T. Differential bile-pancreatic secretory effects of CCK-58 and CCK-8. *Am J Physiol Gastrointest Liver Physiol.* 2004;286(3):G395-402

Rivera JA, Werner J, Warshaw AL, Lewandrowski KB, Rattner DW, Fernandez del Castillo C. Lexipafant fails to improve survival in severe necrotizing pancreatitis in rats. *Int J Pancreatol.* 1998 ;23(2):101-6

Rizzuto R, Pozzan T. Microdomains of intracellular Ca^{2+} : molecular determinants and functional consequences. *Physiol Rev* 2006;86:369-408

Rousset S, Alves-Guerra MC, Mozo J, Miroux B, Cassard-Doulicier AM, Bouillaud F, Ricquier D. The biology of mitochondrial uncoupling proteins. *Diabetes* 2004;53(Suppl 1):S130-5.

Runzi M, Saluja A, Lerch MM, Dawra R, Nishino H, Steer ML. Early ductal decompression prevents the progression of biliary pancreatitis: an experimental study in the opossum. *Gastroenterology*. 1993;105(1):157-64.

Ruthenbürger M, Mayerle J, Lerch MM Cell biology of pancreatic proteases. *Endocrinol Metab Clin North Am*. 2006;35(2):313-31

Sahin-Toth M. The pathobiochemistry of hereditary pancreatitis: studies on recombinant human cationic trypsinogen. *Pancreatology* 2001; 1: 461-465

Saluja A K, E A Donovan, K Yamanaka, Y Yamaguchi, B Hofbauer, M L Steer. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology* 1997;113(1):304-10

Saluja AK, Lerch MM, Phillips PA, Dudeja V. Why does pancreatic overstimulation cause pancreatitis? *Annu Rev Physiol* 2007;69:249-269.

Sanchez-Bernal C, Garcia-Morales OH, Dominguez C, Martin-Gallan P, Calvo JJ, Ferreira L, Perez-Gonzalez N. Nitric oxide protects against pancreatic subcellular damage in acute pancreatitis. *Pancreas*. 2004;28(1):e9-15.

Sandstrom P, Woods CM, Brooke-Smith M, Saccone GT, Toouli J, Svanvik J. Highly selective iNOS inhibition and sphincter of Oddi motility in the Australian possum. *Acta Physiol Scand*. 2004;181(3):321-31.

Sarr MG, Nagorney DM, Mucha P Jr, Farnell MB, Johnson CD. Acute necrotizing pancreatitis: management by planned, staged pancreatic necrosectomy/debridement and delayed primary wound closure over drains. *Br J Surg*. 1991;78(5):576-81.

Satoh A, Gukovskaya AS, Nieto JM, Cheng JH, Gukovsky I, Reeve JR Jr, Shimosegawa T, Pandol SJ. PKC-delta and -epsilon regulate NF-kappaB activation induced by cholecystokinin and TNF-alpha in pancreatic acinar cells. *Am J Physiol Gastrointest Liver Physiol*. 2004;287(3):G582-91

Satoh A, Gukovskaya AS, Reeve Jr JR, Shimosegawa T, Pandol SJ. Ethanol Sensitizes NF- κ B Activation in Pancreatic Acinar Cells through effects on Protein Kinase C Epsilon. *Am J Physiol Gastrointest Liver Physiol*. 2006

Schinder AF, Olson EC, Spitzer NC, and Montal M. Mitochondrial dysfunction is a primary event in glutamate neurotoxicity. *Journal of Neuroscience*, 1996. Volume 16, Issue 19, Pages 6125-6133

Scott SR, Kiessling K, Parekh AB. An examination of the role of intracellular ATP in the activation of store-operated calcium influx and calcium dependent capacitance increases in rat basophilic leukaemia cells. *Pflugers Arch* 1998; 436: 928-33

Schult H, Lorenz W, Maroske D, Lange E, Luben L. Role of histamine in acute pancreatitis *Chir Forum Exp Klin Forsch.* 1979;:269-72.

Sherwood MW, Prior IA, Voronina SG, Barrow SL, Woodsmith JD, Gerasimenko OV, Petersen OH, Tepikin AV. Activation of trypsinogen in large endocytic vacuoles of pancreatic acinar cells. *Proc Natl Acad Sci U S A.* 2007 ; 27;104(13):5674-9.

Shikimi T, Yamamoto D, Handa M. Pancreatic lysosomal thiol proteinases and inhibitors in acute pancreatitis induced in rats. *J Pharmacobiodyn.* 1987;10(12):750-7

Siech M, Weber H, Letko G. Similar morphological and intracellular biochemical changes in alcoholic acute pancreatitis and ischaemic acute pancreatitis in rats. *Pancreas* 1999;14:32-38

Slater TF. Free-radical mechanisms in tissue injury. *Biochem J* 1984; 222: 1-15

Snell R.S. Clinical Anatomy for Medical Students, 1995, Fifth Edition, p222-272, Little, Brown and Company, Boston.

Solovyova N, Veselovsky N, Toescu EC, Verkhatsky A. Ca²⁺ dynamics in the lumen of the endoplasmic reticulum in sensory neurons: direct visualization of Ca²⁺-induced Ca²⁺ release triggered by physiological Ca²⁺ entry. EMBO J 2002;21:622-30.

Song AM, Bhagat L, Singh VP, Van Acker GG, Steer ML, Saluja AK. Inhibition of cyclooxygenase-2 ameliorates the severity of pancreatitis and associated lung injury. Am J Physiol Gastrointest Liver Physiol. 2002; 283(5): G1166-74.

Stigendal L, Olsson R. Alcohol consumption pattern and serum lipids in alcoholic cirrhosis and pancreatitis. A comparative study. Scand J Gastroenterol 1984;19:582-7.

Sun G, Chang TM, Xue WJ, Wey JF, Lee KY, Chey WY. Release of cholecystokinin and secretin by sodium oleate in dogs: molecular form and bioactivity. Am J Physiol 1992;262:G35-43.

Sutton R, Criddle D, Raraty MG, Tepikin A, Neoptolemos JP, Petersen OH. Signal transduction, calcium and acute pancreatitis. Pancreatology. 2003;3(6):497-505

Tando Y, Algul H, Wagner M, Weidenbach H, Adler G, Schmidt RM. Caerulein induced NF-kappaB/ Rel activation requires both calcium and protein kinase C as messengers. *Am J Physiol Gastrointest Liver Physiol* 1999; 277: G678-86

Thorn P, Lawrie AM, Smith PM, Gallacher DV, Peterson OH. Local and global cytosolic calcium oscillations in exocrine cells evoked by agonists and inositol triphosphate. *Cell* 1993; 74 : 661-8

Thrower EC, Diaz de Villalvilla AP, Kolodecik TR, Gorelick FS. Zymogen activation in a reconstituted pancreatic acinar cell system. *Am J Physiol Gastrointest Liver Physiol*. 2005

Tinel H, Cancela J, Gerasimenko JV, Gerasimenko OV, Tepikin AV, Peterson OH. Active mitochondria surrounding the pancreatic acinar granule region prevents spreading of inositol – evoked local cytosolic calcium signals. *EMBO J* 1999; 18: 4999-5008

Toescu EC, O'Neill SC, Petersen OH, Eisner DA. Caffeine inhibits the agonist-evoked cytosolic Ca²⁺ signal in mouse pancreatic acinar cells by blocking inositol trisphosphate production. *J Biol Chem* 1992; 267:23467-70.

Tooze, J., Hollinshead, M., Hensel, G., Kern, H.F., and Hoflack, B. Regulated secretion of mature cathepsin B from rat exocrine pancreatic cells. *Eur. J. Cell Biol.* 1991; 56:187-200

Tsai K, Wang SS, Chen TS, Kong CW, Chang FY, Lee SD, Lu FJ. Oxidative stress: an important phenomenon with pathogenetic significance in the progression of acute pancreatitis. *Gut.* 1998;42(6):850-5.

Tsiotos GG, Luque-de Leon E, Soreide JA, Bannon MP, Zietlow SP, Baerga-Varela Y, Sarr MG. Management of necrotizing pancreatitis by repeated operative necrosectomy using a zipper technique. *Am J Surg* 1998; 175(2):91-8.

Tsukamoto H, Towner SJ, Yu GS, French SW. Potentiation of ethanol-induced pancreatic injury by dietary fat. Induction of chronic pancreatitis by alcohol in rats. *Am J Pathol* 1988;131:246-57.

Uhl W, Warshaw A, Imrie C, Bassi C, McKay CJ, Lankisch PG, Carter R, Di Magno E, Banks PA, Whitcomb DC, Dervenis C, Ulrich CD, Satake K, Ghaneh P, Hartwig W, Werner J, McEntee G, Neoptolemos JP, Buchler MW; International Association of Pancreatology. IAP Guidelines for the Surgical Management of Acute Pancreatitis. *Pancreatology.* 2002;2(6):565-73

Uhlmann D, Ludwig S, Geissler F, Tannapfel A, Hauss J, Witzigmann H. Importance of microcirculatory disturbances in the pathogenesis of pancreatitis. *Zentralbl Chir.* 2001;126(11):873-8.

vanSonnenberg E, Wittich GR, Casola G, Stauffer AE, Polansky AD, Coons HG, Cabrera OA, Gerver PS. Complicated pancreatic inflammatory disease: diagnostic and therapeutic role of interventional radiology. *Radiology.* 1985;155(2):335-40

Vitone LJ, Greenhalf W, Howes NR, Raraty MG, Neoptolemos JP. Trypsinogen mutations in pancreatic disorders. *Endocrinol Metab Clin North Am.* 2006;35(2):271-87.

Voet, Donald; Judith G. Voet, Charlotte W. Pratt. *Fundamentals of Biochemistry*, (2006). 2nd Edition. John Wiley and Sons, Inc.. pp. 547.

Vonlaufen A, Wilson JS, Pirola RC, Apte MV. Role of alcohol metabolism in chronic pancreatitis. *Alcohol Res Health.* 2007;30(1):48-54. Review

Voronina S, Longbottom R, Sutton R, Peterson OH, Tepikin AV. Bile acids induce calcium signals in mouse pancreatic acinar cells: implications for bile-induced pancreatic pathology. *J Physiol* 2002; 540.1: 49-55

Voronina S, Sukhomlin T, Johnson PR, Erdemli G, Peterson OH, Tepikin AV. Correlation of NADH and calcium signals in mouse pancreatic acinar cells. *J Physiol* 2002; 539: 41-52

Voronina SG, Gryshchenko OV, Gerasimenko OV, Green AK, Petersen OH, Tepikin AV. Bile acids induce a cationic current, depolarizing pancreatic acinar cells and increasing the intracellular Na^+ concentration. *J Biol Chem*. 2005;280(3):1764-70

Voronina SG, Sherwood MW, Gerasimenko OV, Petersen OH, Tepikin AV. Visualizing formation and dynamics of vacuoles in living cells using contrasting dextran-bound indicator: endocytic and nonendocytic vacuoles. *Am J Physiol Gastrointest Liver Physiol*. 2007;293(6):G1333-8

Wakui M, Osipchuk YV, Petersen OH. Receptor-activated cytoplasmic Ca^{2+} spiking mediated by inositol trisphosphate is due to Ca^{2+} -induced Ca^{2+} release. *Cell* 1990;63:1025-32.

Walsh JH. Gastrointestinal hormones. In: Johnson LR (ed) *Physiology of the gastrointestinal tract*, 2nd edition, Raven Press, New York, 1987; pp 181-253.

Wang YL, Hu R, Lugea A, Gukovsky I, Smoot D, Gukovskaya AS, Pandol SJ. Ethanol feeding alters death signaling in the pancreas. *Pancreas*. 2006;32(4):351-9.

Ward JB, Sutton R, Jenkins SA, Peterson OH. Progressive disruption of acinar cell calcium signalling is an early feature of caerulein induced pancreatitis in mice. *Gastroenterology* 1996; 111: 481-91

Ward JB, Peterson OH, Jenkins SA, Sutton R. Is an elevated concentration of acinar cytosolic free ionised calcium the trigger for acute pancreatitis? *Lancet* 1995; 346: 1016 - 9

Weber H, Roesner JP, Nebe B, Rychly J, Werner A, Schroder H, Jonas L, Leitzmann P, Schneider KP, Dummmler W. Increased cytosolic Ca^{2+} amplifies oxygen radical-induced alterations of the ultrastructure and the energy metabolism of isolated rat pancreatic acinar cells. *Digestion*. 1998;59(3):175-85.

Werner J, Laposata M, Fernandez-del- Castillo C, Saghir M, Iozzo RV, Lewandrowski KB, Warshaw AL. Pancreatic injury in rats induced by fatty acid ethyl ester, a nonoxidative metabolite of alcohol. *Gastroenterology* 1997; 113(1): 286-294

Werner J, Saghir M, Castillo CFD, Warshaw AL, Laposata M. Linkage of oxidative and non oxidative ethanol metabolism in the pancreas and toxicity of nonoxidative ethanol metabolites for pancreatic acinar cells. *Surgery* 2001;129(6): 736-744

Werner J, Saghir M, Warshaw AL, Lewandrowski KB, Laposata M, Iozzo RV, Carter EA, Schatz RJ, Fernández-Del Castillo C. Alcoholic pancreatitis in rats: injury from nonoxidative metabolites of ethanol. *Am J Physiol Gastrointest Liver Physiol*. 2002 ;283(1):G65-73

Whitcomb DC, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, Martin SP, Gates LK Jr, Armann ST, Toskes PP, Liddle R, McGrath K, Uomo G, Post JC, Ehrlich GD. Hereditary Pancreatitis is caused by a mutation in the cationic trypsinogen gene. *Nat Genet* 1996; 14: 141-5

Williams JA. Intracellular signalling mechanisms activated by cholecystokinin – regulating synthesis and secretion of digestive enzymes in pancreatic acinar cells. *Ann Rev Physiol* 2001; 63: 77-97

Wilson JS, Apte MV. Role of alcohol metabolism in alcoholic pancreatitis. *Pancreas*. 2003;27(4):311-5

Wilson JS, Korsten MA, Pirola RC. Alcohol-induced pancreatic injury (Part I). Unexplained features and ductular theories of pathogenesis. *Int J Pancreatol*. 1989 4(2):109-25.

Wilson JS, Bernstein L, McDonald C, Tait A, McNeil D, Pirola RC. Diet and drinking habits in relation to the development of alcoholic pancreatitis. *Gut* 1985;26:882-7.

Winslet M, Hall C, London NJ, Neoptolemos JP. Relation of diagnostic serum amylase levels to aetiology and severity of acute pancreatitis. *Gut*. 1992 Jul;33(7):982-6.

Working Party on Acute Pancreatitis. UK guidelines for the management of acute pancreatitis. *Gut* 2005;54:iii1-iii9

Yadav D, Pitchumoni CS. Issues in hyperlipidemic pancreatitis. *J Clin Gastroenterol* 2003;36:54-62.

Yamagiwa T, Shimosegawa T, Satoh A, Kimura K, Sakai Y, Masamune A. Inosine alleviates rat caerulein pancreatitis and pancreatitis-associated lung injury. *J Gastroenterol*. 2004;39(1):41-9.

Yamamoto M, Reeve JR Jr, Keire DA, Green GM. Water and enzyme secretion are tightly coupled in pancreatic secretion stimulated by food or CCK-58 but not by CCK-8. *Am J Physiol Gastrointest Liver Physiol*. 2005;288(5):G866-79.

Yang R, Uchiyama T, Alber SM, Han X, Watkins SK, Delude RL, Fink MP. Ethyl pyruvate ameliorates distant organ injury in a murine model of acute necrotizing pancreatitis. *Crit Care Med*. 2004; 32(7): 1453-9.

Yoo SH, So SH, Kweon HS, Lee JS, Kang MK, Jeon CJ. Coupling of the inositol 1,4,5-triphosphate receptor and chromogranins A and B in secretory granules. *J Biol Chem* 2000; 275:12553-9

Zhang Y, Rodney GG, Schneider MF. Effects of azumolene on Ca^{2+} sparks in skeletal muscle fibers. *J Pharmacol Exp Ther*. 2005;314(1):94-102

Statement of Originality/ Permissions

The work described in this thesis was carried out while I was employed as a Research Registrar within the Division of Surgery and Oncology and Department of Physiology, University of Liverpool, between January 2004 and January 2007.

The vast majority of work presented here has been carried out by me except for some experiments demonstrated in the results of Chapter 3 which was produced by colleagues in the laboratory – Drs O. V. Gerasimenko and M. Chvanov.

The work, detailed below, has been reproduced with permission from the individual who performed the experiment.

Chapter 3:

1) Figure 3.6 (page 160)

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2) Figure 3.8 (page 168)

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Appendix

Publications arising from this period of research

Booth DM, **Murphy JA**, Mukherjee R, Neoptolemos JP, Gerasimenko OV, Tepikin AV, Petersen OH, Sutton R, Criddle DN.

Reactive oxygen species induced by bile acid elicit apoptosis and protect against pancreatic acinar cell necrosis.

Gastroenterology. 2011;140(7):2116-25

Murphy JA, Criddle DN, Sherwood M, Chvanov M, Mukherjee R, McLaughlin E, Booth D, Gerasimenko JV, Raraty MG, Ghaneh P, Neoptolemos JP, Gerasimenko O, Tepikin AV, Green GM, Reeve JR, Petersen OH, Sutton R.

Direct activation of cytosolic Ca²⁺ signaling and enzyme secretion by cholecystokinin in human pancreatic acinar cells.

Gastroenterology. 2008;135(2):632-41

Criddle DN, McLaughlin E, **Murphy JA**, Petersen OH, Sutton R.

The pancreas misled: signals to pancreatitis.

Pancreatology. 2007;7(5-6):436-46

Criddle DN, **Murphy J**, Fistetto G, Barrow S, Tepikin AV, Neoptolemos JP,
Sutton R, Petersen OH.

Fatty acid ethyl esters cause pancreatic calcium toxicity via inositol trisphosphate
receptors and loss of ATP synthesis.

Gastroenterology. 2006;130(3):781-93

Raraty MG, **Murphy JA**, Mcloughlin E, Smith D, Criddle D, Sutton R.

Mechanisms of acinar cell injury in acute pancreatitis.

Scand J Surg. 2005;94(2):89-96