THE PATHOLOGICAL ROLE OF BCL-2 FAMILY PROTEINS AND THE PROTECTIVE ROLE OF CALCIUM REGULATOR CALMODULIN IN PANCREATIC ACINAR CELLS

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

Shuang Peng

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ABSTRACT

Acute pancreatitis is a life threatening illness characterized by premature activation of digestive enzymes inside pancreatic acinar cells. This leads to necrosis and digestion of the pancreatic tissue and its surroundings. As of yet, no specific therapy to treat acute pancreatitis has been found.

The physiological and pathological processes are controlled to a large extent by calcium signalling in pancreatic acinar cells. Intracellular calcium signalling not only regulates the expression and secretion of digestive enzymes but also contributes to the activation of trypsinogen, which eventually leads to pancreatitis and causes necrosis of pancreatic tissue.

The Bcl-2 protein is a member of Bcl-2 family proteins that regulate programmed cell death. It has been shown that Bcl-2 proteins are engaged in the regulation of cellular calcium homeostasis. One aim of this study was to investigate the effects of the anti-apoptotic Bcl-2 protein on intracellular calcium fluxes. Another goal was to study the effects of calmodulin activators (calcium-like peptides) on pathophysiological processes in pancreatic acinar cells.

Our data showed that thapsigargin and acetylcholine-induced cytosolic calcium release was quicker in pancreatic acinar cells lacking Bcl-2 protein than in wild type cells. We blocked the endoplasmic reticulum Ca^{2+} pumps by thapsigargin and removed the external Ca^{2+}, therefore, Ca^{2+} extrusion was the only process restoring the basal cytosolic Ca^{2+} level. Our results show that cells lacking Bcl-2 extrudes Ca^{2+} more efficiently than the wild type cells. Furthermore, Ca^{2+} extrusion was decreased in the pancreatic cancer cell line AR42J which has overexpression of Bcl-2. This effect can be explained by the increased activity of plasma membrane calcium-activated ATPase (PMCA) which was blocked by Caloxin 3A1, which in turn promotes necrosis in pancreatic acinar cells.

In addition, the data presented here showed that the calmodulin activators CALP-3 and CALP-1 did not affect the physiological calcium signalling in pancreatic acinar cells but instead contributed to either increasing the cell survival rate or causing apoptosis. CALPs were applied to specifically activate calmodulin which reduced the toxic effects of pancreatic acinar cells challenged with alcohol or the oxidant menadione.

This study shows that the inhibition of calcium recovery mechanisms by Bcl-2 is significantly pathological. The study also proposes that calcium-like peptides could potentially be developed as therapeutic agents for acute pancreatitis.

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ABBREVIATIONS

$[Ca^{2+}]$	calcium concentration
ACh	acetylcholine
ADP	adenosine 5'-diphosphate
AM	acetoxymethyl Apaf-1 Apoptotic protease activating factor 1
Apaf1	apoptotic protease activating factor-1
ATP	adenosine 5'-triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 antagonist / killer
Bcl-2	B-cell leukaemia / lymphoma 2
Bcl-2 KO	B-cell leukaemia / lymphoma 2 knockout
Bcl-w	Bcl-2-like protein 2
Bcl-xL	B-cell lymphoma - extra large
BH	Bcl-2 Homology
Bid	BH3 interacting domain death agonist
Bik	Bcl-2-interacting killer
Bim	Bcl-2-interacting mediator of cell death
cADPR	cyclic adenosine diphosphate ribose
CALP	Ca^{2+}-like peptides
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CICR	calcium induced calcium release
CPA	cyclopiasonic acid
DMSO	Dimethyl sulfoxide
EDTA	ethylene diamine tetraacetic acid
EF-hand	calcium binding helix-loop-helix structural domain
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
EtOH	ethanol
FAEE	fatty acid ethyl esters
FBS	foetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IP_3	inositol 1,4,5-triphosphate
IP_3R	inositol 1,4,5-triphosphate receptor
MPTP	mitochondrial permeability transition pore
NAADP	nicotinic acid adenine dinucleotide phosphate
NCX	Na^{+}-Ca^{2+} exchanger
PBS	phosphate buffered saline
PLC	phospholipase C
PMCA	plasma membrane calcium-activated ATPase

POAEE	palmitoleic acid ethyl ester
PTP	permeability transition pore
PUMA	p53 upregulated modulator of apoptosis
ROS	reactive oxygen species
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RyR	ryanodine receptor
SERCA	sarcoplasmic/endoplasmic reticulum calcium ATPase
SOCE	store operated calcium entry
t-Bid	truncated Bid
TBS	Tris buffered saline
Tg	thapsigargin
WT	wild type

Publication

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CHAPTER 1:

INTRODUCTION

Chapter 1: Introduction

1.1. Pancreas

In humans, the pancreas (Fig. 1.1) is the second biggest gland. Generally, the length of the pancreas is about 17~20cm, the width is about 3~5cm, the thickness is about 1.5~2.5cm, and its weight ranges from 82 to117g. It is divided into four parts: the head, the neck, the body and the tail. The head of the pancreas, the largest of the four, is embedded in the duodenal loop. The neck is located anterior to the superior mesenteric vein. The body of the pancreas located between the neck and the tail and its posterior edge is close to the lumbar region. The tail of the pancreas is a thin and narrow part to the left of the pancreas. The tail of pancreas is surrounded by serosa whereas the other parts are found in the retro-peritoneal space. Therefore the lesions of the pancreas are often deep and hidden.

The pancreas has both exocrine and endocrine functions. The exocrine functions are performed by the pancreatic acini and the duct. The pancreatic acinar cells secrete pancreatic juice which contains sodium bicarbonate, trypsin, lipase, amylase and other digestive enzymes. This pancreatic fluid passes to the duodenum via the pancreatic duct. The digestive enzymes of the pancreatic fluid can help to further break down the carbohydrates, proteins, and lipids (fats) in the chyme. The secretion of pancreatic fluid is controlled by humoral regulation and, to a lesser extent, the parasympathetic nerves.

In contrast, the endocrine functions are performed by the islets of Langerhans, which are cell clusters varying in size and shape and are distributed among the pancreatic acinar cells. There are approximately one million islets in the pancreas, and they are mainly located in the body and the tail of the pancreas. The islet cells have different functions; β cells secrete insulin while the less common glucagon-secreting α cells are also involved in blood glucose homeostasis. The other cells include δ cells which secrete somatostatin and PP cells which secrete pancreatic polypeptide in order to regulate pancreatic secretion.

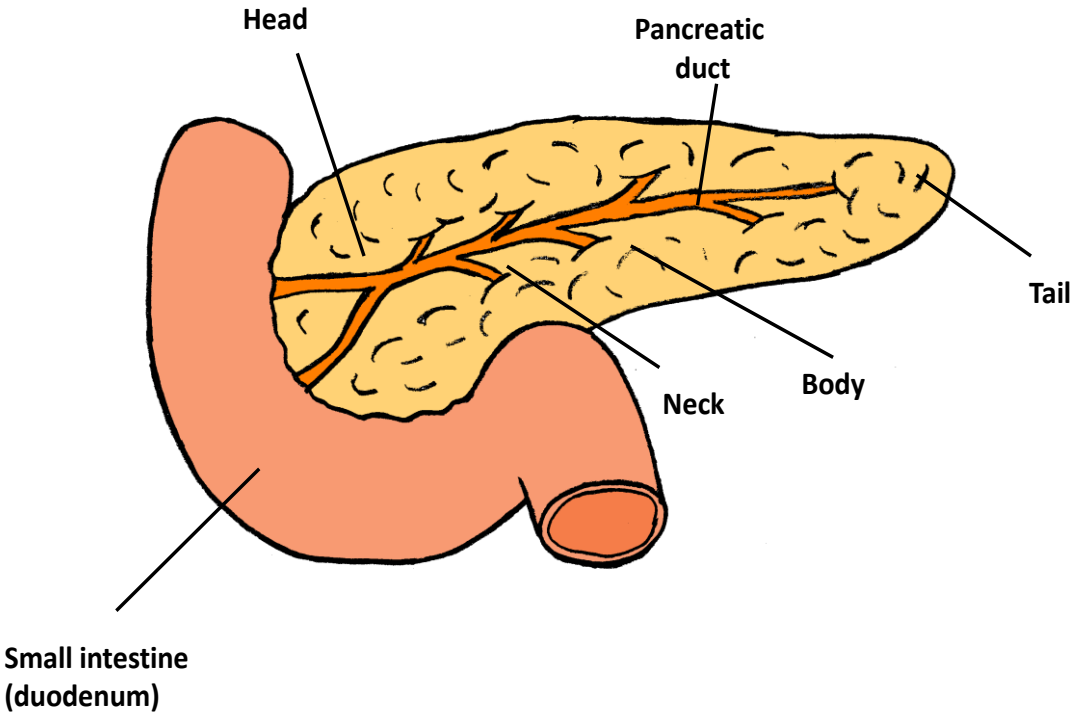


Fig. 1.1. Schematic illustration of the human pancreas
See full description in text. (Unpublished)

1.2. Pancreatic acinar cells

The acinar cells account for 82% of the pancreas; the other parts are duct cells, blood vessels, endocrine cells and the extracellular matrix [1]. Each pancreatic acinar cell is polarized with two visible areas: basolateral pole and apical pole (Fig. 1.2). They are separated by the mitochondrial belt which can buffer the calcium from the apical pole to the basolateral pole. The basolateral pole is larger (even up to 90% of the cell volume) than the apical pole, and the majority of the endoplasmic reticulum (ER) is located in basolateral area. However, the endoplasmic reticulum can sometimes penetrate the mitochondria belt into the apical pole. The apical pole contains zymogen granules (ZGs) where digestive proenzymes are stored.

One acinar cell alone cannot perform an exocrine function in the pancreatic tissue, which is why the acinar cells are organized into acini. The acini are linked by a large number of gap junctional channels which allow both direct electrical and chemical intracellular communication [2, 3]. The main function of acinar cells is to secrete digestive enzymes in response to eating food.

This secretion is mediated by acetylcholine (ACh), which is released from the endings of the vagus nerve, and the circulating hormone cholecystokinin (CCK). The secretion process itself takes place by exocytosis. The granule membrane fuses with the apical (luminal) cell membrane and subsequently the opening pole of the granule allows zymogens to move into the acinar lumen. The acinar cells and the small ducts each secrete a neutral Cl^- rich and a HCO_3^- rich fluid respectively so that the zymogens can move from the duct system into the gut.

The physiological functions of pancreatic acinar cells are controlled by calcium. Different patterns of cytosolic calcium responses differentiate between physiology and pathology. Physiological doses of ACh and CCK evoke either small cytosolic $[Ca^{2+}]$ oscillations, which are often confined only to apical area of the cell or global calcium waves that originate in the apical area and spread toward the basolatoral area. ACh and CCK activate the precursor enzymes trypsinogen, chymotrypsinogen and procarboxypeptidases, secreted by acinar cells. The ACh or CCK receptors at the basolateral plasma membrane receive stimuli for secretion (such as acetylcholine or cholecystokinin) leading to the generation of a second messenger response, which in turn liberates calcium from the intracellular stores.

Inositol 1,4,5-trisphosphate receptors (IP_3Rs) and ryanodine receptors (RyRs) expressed in the ER of the pancreatic acinar cells are located in different areas of the cell. IP_3Rs are predominantly present in the apical pole of the pancreatic acinar cell [4, 5], whereas RyRs are evenly distributed at the ER membranes in the apical as well as basolateral region [6, 7]. Despite the stimulus acting on the basolateral membrane, the calcium response originates in the apical pole of the cell. Accumulation of both IP_3Rs and RyRs in the granular area is associated with increased sensitivity of this region for

calcium-induced calcium release (CICR). CICR involves a small increase in cytosolic [Ca^{2+}] which induces further release of calcium from the intracellular stores via activation of additional calcium channels, as compared to the basolateral pole.

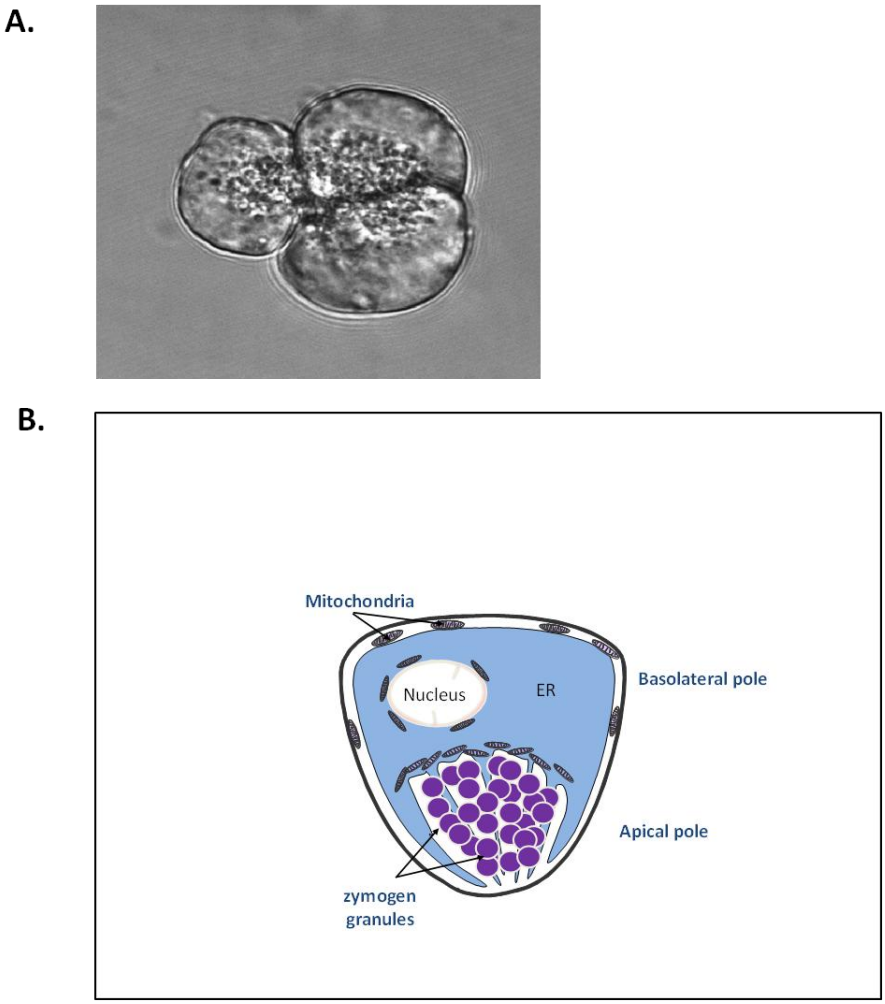


Fig. 1.2. Structure of a pancreatic acinar cell
(A) Transmitted light image from confocal microscope showing a typical pancreatic acinar cell isolated from C57BL6 mouse pancreas. (Unpublished data). (B) Schematic illustration of the pyramid-shaped structure of a pancreatic acinar cell. The basolateral pole contains most of the ER; zymogen granules are stored in the apical pole. [Adapted from Gerasimenko JV]. [8]

1.3. Calcium signalling in pancreatic acinar cells

Generally, the physiological cytosolic calcium concentration is between 0.1 μM and 1 μM in pancreatic acinar cells. Early studies indicated that the ER was the principal intracellular calcium store and the neurotransmitters acetylcholine (ACh) as well as the circulating hormone cholecystokinin (CCK) elicited Ca^{2+} release from the ER [9-11]. Different patterns of cytosolic calcium responses differentiate between

physiology and pathology in pancreatic acinar cells. The physiological role of Ca^{2+} signals is to trigger enzyme secretion via exocytosis [12, 13]. However, calcium signals can also be dangerous for pancreatic acinar cells in the specific situation when proenzymes are inappropriately activated inside the acinar cells before secretion.

As mentioned before, small cytosolic [Ca^{2+}] oscillations or global calcium waves evoked by ACh or CCK are the physiological calcium signalling events. However, excessive doses of ACh or CCK or treatment with menadione (induction of oxidative stress) [14, 8] cause global large sustained elevations of cytosolic [Ca^{2+}], which are toxic and can lead to cell death (Fig. 1.3.A-B) [15, 16].

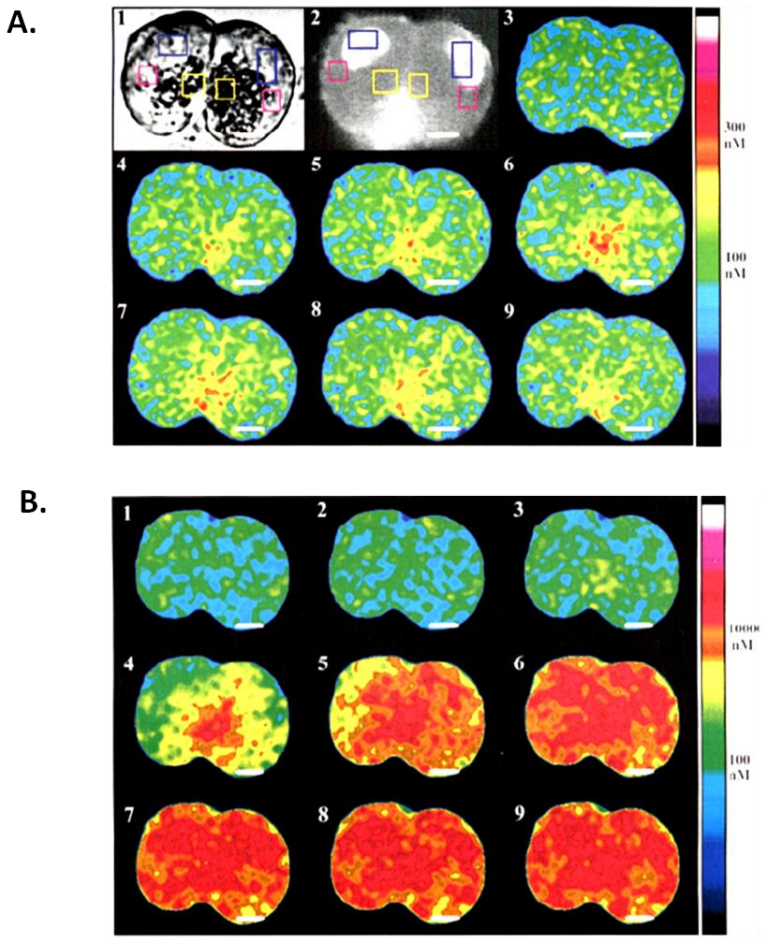


Fig. 1.3. Physiological and pathological calcium responses in pancreatic acinar cells

(A) Physiological calcium responses to small doses of ACh in pancreatic acinar cells are restricted to the granular area [Adapted from Gerasimenko OV, 1996]. [17] (B) Excessive doses of ACh induce sustained global Ca^{2+} rise in the cytosol of pancreatic acinar cells [Adapted from Gerasimenko OV, 1996]. [17]

The physiological stimulants ACh and CCK act on the outside of the acinar plasma membrane [18, 19]. ACh binds to muscarinic receptors which leads to the activation

of phospholipase C (PLC) and the subsequent generation of inositol 1,4,5-trisphosphate (IP_3), an intracellular messenger, releasing Ca^{2+} from the ER [20]. CCK activates the enzyme ADP-ribosyl cyclase by a yet unknown mechanism to form two separate messengers NAADP (nicotinic acid adenine dinucleotide phosphate) and cADPR (cyclic ADP-ribose). The three messengers, IP_3, cADPR, and NAADP, can liberate Ca^{2+} from the ER by activating IP_3Rs and RyRs, respectively (Fig. 1.4).

The initial Ca^{2+} signal elicited by either ACh or CCK is due to Ca^{2+} release from internal stores and the initial enzyme secretory response does not require external Ca^{2+} [21]. However Ca^{2+} entry from the external solution is indispensable for the sustained elevation of $[Ca^{2+}]$i and sustained enzyme and fluid secretion [14,21]. The mechanism of store-operated Ca^{2+} entry is still elusive and under intensive investigation.

The termination of Ca^{2+} signals, caused by the closure of Ca^{2+} release channels, is as important as their initiation. When $[Ca^{2+}]$ is released from the ER ($[Ca^{2+}]_{ER}$) into the cytoplasm due to sustained supramaximal ACh stimulation, the excess calcium is pumped out of the cell by the plasma membrane Ca^{2+} pump (plasma-membrane-Ca^{2+}-activated-ATPase, or PMCA) [22]. The calcium then re-enters the cell via store-operated Ca^{2+} channels and is taken up into the ER [23, 24]. Discontinuation of supramaximal ACh stimulation immediately causes Ca^{2+} pump (sarco-endoplasmic-reticulum-Ca^{2+}-activated ATPase, or SERCA)-mediated Ca^{2+} reuptake into the ER and a rapid reduction of $[Ca^{2+}]_i$ back to the resting level, whereas it takes much longer for $[Ca^{2+}]_{ER}$ to recover fully [23]. $[Ca^{2+}]_{ER}$ seems to exert a more important control on the ER Ca^{2+} uptake than $[Ca^{2+}]_i$.

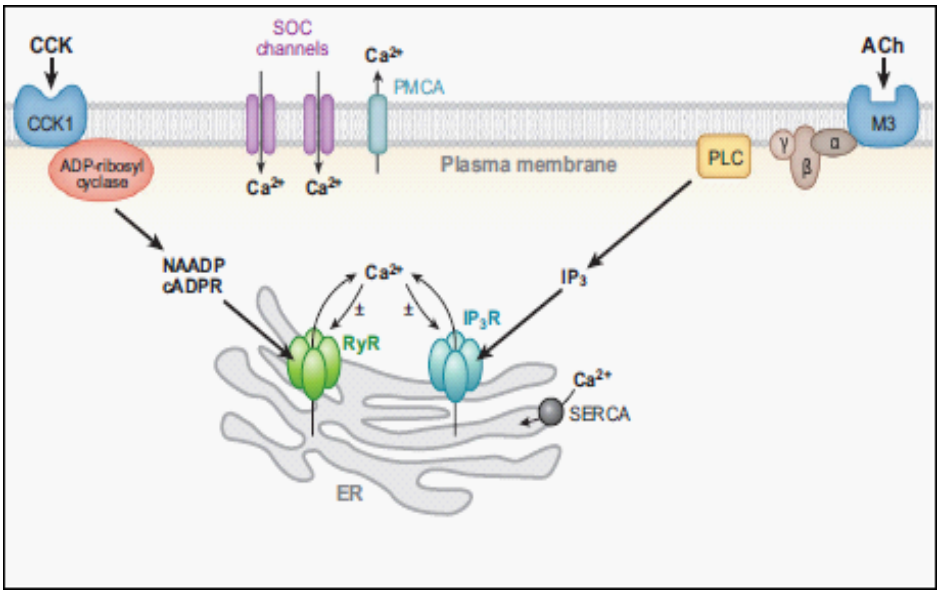


Fig. 1.4. Calcium signalling in pancreatic acinar cells
Schematic illustration of calcium signalling events occurring at the plasma membrane,

and the ER membrane [Adapted from Petersen OH 2008]. [25]

1.4 Acute Pancreatitis

Acute pancreatitis is a life-threatening disease characterized by premature activation of digestive enzymes inside pancreatic acinar cells which leads to high levels of necrosis and digestion of the pancreatic tissue and its surroundings. There is so far no specific therapy for the disease [26]. A large proportion of acute pancreatitis is caused by bile duct stones or excessive alcohol intake, with alcohol being a major cause of chronic pancreatitis in western countries. The prevalence of acute pancreatitis is up to 1 in 1,000 people per year, with severe complications developing in approximately 20% of patients diagnosed with the disease. Complications include agonizing pain, extensive inflammation and subsequent necrosis of pancreatic and surrounding tissue, multiple organ failure, and long hospitalization and in up to 5% of cases mortality.
Repeated cases of acute pancreatitis often lead to chronic pancreatitis, markedly increasing the risk for development of pancreatic cancer. Pancreatic cancer, though only the fifth most common cause of death in cancer patients, is the most deadly with a very high 5-year mortality rate [16, 26].

Gallstones can cause acute pancreatitis, because they can block the common (bile–pancreatic) channel and would allow bile to reflux to the pancreas, causing injury to the pancreatic acinar cells. This particular theory still remains controversial [27], but Ca^{2+}-dependent cell death caused by bile acid uptake has been demonstrated in pancreatic acinar cells in vitro [28].

Although alcohol abuse is known to be associated with acute pancreatitis, the number of people who drink alcohol excessively and develop acute pancreatitis is in the minority (<10%) [29]. How can this be explained? Alcohol itself, even in very high concentrations, usually has only mild effects on cellular Ca^{2+} homeostasis. However, it has been shown that a combination of alcohol and fatty acids (fatty acid ethyl esters) causes massive intracellular Ca^{2+} release and acute trypsin activation in freshly isolated pancreatic acinar cells. It has also been indicated that the intake of ethanol with a high fat diet causes inflammation of pancreas in vivo experiments [30, 31]. It is believed that the extent of pancreatic acinar cell injury is determined by the non-oxidative metabolites of ethanol - fatty acid ethyl esters (FAEEs) [32].

The current consensus of the initiation step of acute pancreatitis is that proteases are activated inside the pancreatic acinar cell. This process relies on Ca^{2+} release from the intracellular stores (ER and acidic store) followed by Ca^{2+} entry from the external space. It has also been found that intracellular vacuolization, which involves the zymogen granules being transformed into empty-looking vacuoles, happens at the same time as the protease activation [33].

1.5 Apoptosis and necrosis - major types of cell death in pancreas

Cell death, defined as an irreversible loss of membrane integrity [34], plays a crucially important role in animal and plant development. Up to 11 types of cell death have been classified so far [35]. In the pancreatic acinar cell there are two principal types of cell death: apoptosis and necrosis. Apoptosis is genetically regulated and occurs via both caspase-dependent and independent pathways [36], while necrosis is thought to be largely non-programmed and uncontrolled, although this view has recently been debated [37]. Ca^{2+} signalling has been linked to both apoptotic and necrotic cell death [38]; in the pancreatic acinar cell oscillatory global rises of cytosolic Ca^{2+} may induce apoptosis [14], while sustained elevations promote necrosis [39, 40]. It has been accepted for many years that necrosis is the major form of pancreatic acinar cell death [41]. Current evidence suggests that the balance between apoptosis and necrosis may influence the severity of acute pancreatitis [42, 43].

Cells that die accidentally in response to an acute insult, such as trauma or a lack of blood supply, usually do so by necrosis. Necrotic cells swell and burst, spilling their contents over their neighbours, eliciting an inflammatory response in the surrounding pancreatic tissue. Activation and infiltration of neutrophils contribute to an increase in intracellular digestive enzyme activation, thus aggravating acute pancreatitis [44].

It has been established that acute pancreatitis is caused by CCK in all species [45]. Experimental acute pancreatitis is induced by the CCK analogue cerulean [46]. Mice were repeatedly injected with cerulein and subsequently freshly isolated pancreatic acinar cells were obtained from them. It was found that abnormal secretagogue-induced Ca^{2+} signals were recorded in these cells, which suggests that the normal homeostatic control became compromised [47]. Furthermore, high doses of CCK induce elevation of baseline Ca^{2+} in isolated pancreatic acinar cells. This elevation is strongly associated with the premature intracellular activation of trypsinogen, the hallmark of acute pancreatitis [33, 48]. Other pathological stimuli which cause pancreatic injury can also raise cytosolic Ca^{2+}. They include non-oxidative ethanol metabolites [39, 40], duct ligation [49] and bile acids [28].

In contrast to necrosis, apoptosis is a form of programmed cell death. It is by far the most common and best understood form. It is known that in apoptosis, cell deaths are ‘suicides’, whereby the cells activate an intracellular death program and kill themselves in a controlled way. There are specific morphological changes which cells undergo in the process of apoptosis. They shrink and condense, the cytoskeleton collapses, the nuclear envelope disassembles, and the nuclear chromatin condenses and breaks up into fragments. However, the typical event of apoptosis is that phosphatidylserine moieties flip from the inner to the outer side of the plasmalemmal membrane. It can be experimentally detected with the help of Annexin V staining using fluorescence microscopy [8, 50].

During the process the cell surface often blebs and, if the cell is large, it often breaks

up into membrane-enclosed fragments called apoptotic bodies. In this way, the cell dies neatly and is rapidly cleared away because the surface of the cell or apoptotic bodies become chemically altered, allowing a neighbouring cell or a macrophage (a specialized phagocyte) to swallow them before they spill their contents, and thus preventing a damaging inflammatory response.

Apoptosis occurs by two principal routes: via extrinsic (receptor-mediated) and intrinsic (classical: mitochondrial) pathways [38]. Caspases, aspartate-specific cysteine proteases, are the key factor of the initiation and execution of programmed cell death in apoptotic pathways. There are at least 14 isoforms of caspases [51]. Caspases are divided into 'initiators' (caspases2, 8, 9 and 10), which induce the proteolytic cascade that results in activation of 'executioners' (caspases 3, 6 and 7), which cleave numerous target proteins. However, only the intrinsic pathway is tightly regulated by the Bcl-2 family proteins.

Activation of the intrinsic pathway alters mitochondrial structure and function. It is believed that this change initiates permeabilization of the outer mitochondrial membrane which leads to the formation of the permeability transition pore (PTP). As a result, apoptogenic factors such as cytochrome c, are released from the mitochondria [52].

It is known that Ca^{2+} regulates the intrinsic apoptotic pathway [53]. Ca^{2+}-dependent opening of the mitochondrial permeability transition pore (MPTP) is deemed crucial for cytochrome c release into the cytosol [54]. Once cytochrome c is released, apoptotic protease activating factor-1 (Apaf1) and pro-caspase 9, which are activated by apoptotic peptidase, combine to form an apoptosome that activates caspase 9. Subsequently, it activates caspase 3 that cleaves specific apoptotic targets causing cell death [55].

However, recent evidence has suggested that formation of the MPTP and cytochrome c release in cells may occur without isoforms of the adenine nucleotide translocator (ANT). The pore was no longer regulated by ANT ligands, while higher Ca^{2+} was required for permeability transition [56].

1.6 Role of Bcl-2 family proteins

As mentioned earlier, the Bcl-2 (B-cell lymphoma 2) family proteins regulate the intrinsic pathway of apoptosis and are the major group of intracellular regulators of apoptosis. There are a total of 20 or so members in the Bcl-2 family known to date. The proteins of the Bcl-2 family encoded by the Bcl2 gene regulate mitochondrial outer membrane (MOM) permeabilization that releases cytochrome c and other intermembrane mitochondrial proteins into the cytosol. This activates the downstream caspase cascade and is thought to be an irreversible point for cell death. Hence, the Bcl-2 family proteins are crucial regulators of the mitochondrial pathway of

apoptosis.

According to their function in apoptosis and structure, they are grouped into three sub-families based on the number of BH (Bcl-2 Homology) domains they share: (1) anti-apoptotic proteins which possess four BH domains -BH1-4 (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, and A1); (2) pro-apoptotic proteins possessing three BH (BH1-3) domains (Bax, Bak, and Bok); (3) BH3-only proteins that share the common motif named the BH3 domain (Bad, Bid, Bik, Bim, Puma, Noxa, Hrk etc). The anti-apoptotic proteins can bind with the pro-apoptotic proteins to form heterodimers. In this way, they inhibit their respective functions and thereby affect the survival of the cell.

The anti-apoptotic Bcl-2 proteins are mainly located in the outer mitochondrial membrane, the ER and the nuclear envelope. They keep the integrity of the membrane of these organelles in order to prevent the release of intermembrane proteins. For example, they bind to Bak and prevent its oligomerization in the outer mitochondrial membrane; thereby preventing cytochrome c release.

The BH3-only proteins mediate the inhibition of the intrinsic pathway in apoptosis. They are further subdivided into ‘sensitisers/derepressors’ and ‘direct activators’. The ‘sensitisers/derepressors’ such as Noxa, Bim or Bik can only antagonise the function of anti-apoptotic members. The ‘direct activators’ such as Bid and Bim in addition are able to activate Bax and Bak directly. Bcl-2 family proteins constitute an intricate regulatory network that controls cell fate in response to different stimuli.

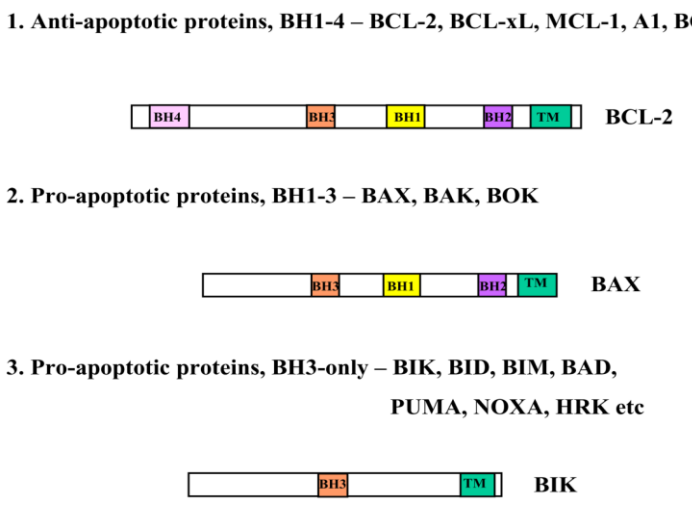


Fig .1.5. Schematic domain structure of Bcl-2 family proteins
Anti-apoptotic proteins posses BH1 - BH4 domains; pro-apoptotic proteins have three BH domains: BH1 - BH3; and BH3-only proteins are characterised by only one BH3 domain. [Adapted from G. Chinnadurai 2008]. [57]

Bcl-2 is the founding member of the Bcl-2 family. Bcl-2 derives its name from B-cell lymphoma 2, as it is the second member of a range of proteins initially described in

chromosomal translocations involving chromosomes 14 and 18 in follicular lymphomas.

However, there is much that is still unknown regarding how Bcl-2 proteins regulate cell death. Several mechanisms have been proposed to explain the inhibition of apoptosis by Bcl-2 proteins. It seems clear that the anti-apoptotic members of the family can block apoptosis by binding directly to the executioners Bax and Bak and to the inducer BH3-only proteins. However, the hierarchy of the interactions that are responsible for apoptosis inhibition remain controversial. Do the anti-apoptotic members inhibit apoptosis mainly by sequestering and blocking the BH3-only proteins, or mostly by binding to Bax and Bak and inhibiting their permeabilization of the membrane? In a recent study using tBid (truncated BH3 interacting domain death agonist) chimeras with different affinities to prosurvival Bcl-2s, Llambi et al [58]. reported that although both inhibitory mechanisms happen in the cell, inhibition of MOM permeabilization by the prosurvival Bcl-2 members via sequestration of the BH3-only proteins is less efficient than via inhibition of Bax and Bak.

A clear impetus in the study of Ca^{2+} homeostasis in apoptosis came from the observation that the important regulatory proteins of apoptosis, the proteins of the Bcl-2 family, are localized in organelles significantly involved in Ca^{2+} handling (in the mitochondria and the ER). Since the ER is the major intracellular calcium store, Bcl-2 proteins have been proposed to be engaged in the regulation of cellular calcium homeostasis. For example it has been observed that Bcl-2 overexpression decreases Ca^{2+} concentration in the ER and diminishes Ca^{2+} entry to this cell compartment [59, 60]. However, other studies support the opposite effects of Bcl-2 on ER calcium: preservation of ER calcium by upregulation of the SERCA [61]. What is more, the interaction between Bcl-2 and IP_3Rs is well established and is believed to inhibit calcium release from the ER [62].

1.7 Aims of the study

One aim of this study is to investigate how the Bcl-2 protein affects Ca^{2+} extrusion in the pancreas. Their mitochondria-related actions are well reported; however the involvement of Bcl-2 proteins in the control of ER calcium levels and calcium fluxes in the cell is still unclear. Therefore this study endeavours to determine potential roles of the Bcl-2 proteins in the regulation of calcium pumps and/or channels as well as their involvement in the passive calcium leak from intracellular stores. Investigation of Bcl-2-regulated calcium signalling pathways might shed more light on our understanding of both physiological and pathological processes in pancreatic acinar cells and help to develop new therapeutic approaches against pancreatic cancer and pancreatitis.

The other aim of this study is to investigate the protective effect of the calmodulin activators – calcium like peptides (CALPs) in pancreatic acinar cells. Alcohol and

oxidative stress can affect calcium signalling in pancreatic acinar cells and contribute to cell death. Calmodulin is a very important calcium sensor which can regulate calcium homeostasis in pancreatic acinar cells. CALPs were applied to exert specific preactivation of calmodulin in order to reduce the toxic effects of pancreatic acinar cells when they were challenged with alcohol or oxidant menadione. There is therefore a possibility of using a calmodulin activator as a potential drug to treat acute pancreatitis.

CHAPTER 2:

MATERIALS AND METHODS

Materials and Methods

1. Reagents and materials

Fluorescent dyes for calcium measurement fura-2, AM and Fluo-4,AM were obtained from Molecular Probes, Invitrogen. Thapsigargin, ionomycin, EGTA were supplied by Calbiochem. Collagenase (various batches) for tissue digestion came from Worthington. Cover glass 32 x 32 mm, thickness No 1 and cover glass 13 mm diameter thickness No 1 were supplied by VWR International. Poly-L-lysine 0.01 % solution and acetylcholine chloride came from Sigma; and calcium chloride was from Fluka.

Tissue culture media and reagents such as RPMI 1640 medium, FBS, Fungizone, Trypsin-EDTA 0.05%, and MEM Amino Acids were obtained from GIBCO. PBS was bought from Cambrex. 1 M HEPES solution was purchased from Lonza, Walkersville, MD, USA. PromoFectin 2000 transfection reagent came from PromoKine. Antibiotic Antimycotic Solution 100x containing 10,000 units/ml penicillin, 10 mg/ml streptomycin and 25 µg/ml amphotericin B were obtained from Sigma.

Wild type C57BL6/J male mice and transgenic mice B6.129S2-BCL-2 (stock no: 002265) were obtained from Jackson Laboratories and were bred in house. Fig. 2.1 shows photographs of a wild type C57BL6/J mouse and a Bcl-2 KO mouse. Comparing with WT mice, we found that Bcl-2 KO mice (Bcl-2 nulls) are characterised by growth retardation and early mortality postnatal. They have small external ears and immature front head features. Because severe polycystic kidney disease causes renal failure, the smallest individuals often become ill and die around the 3rd – 4th week. Another typical trait of the nulls is hair pigmentation loss making their coat colour turn gray with the second follicle cycle.

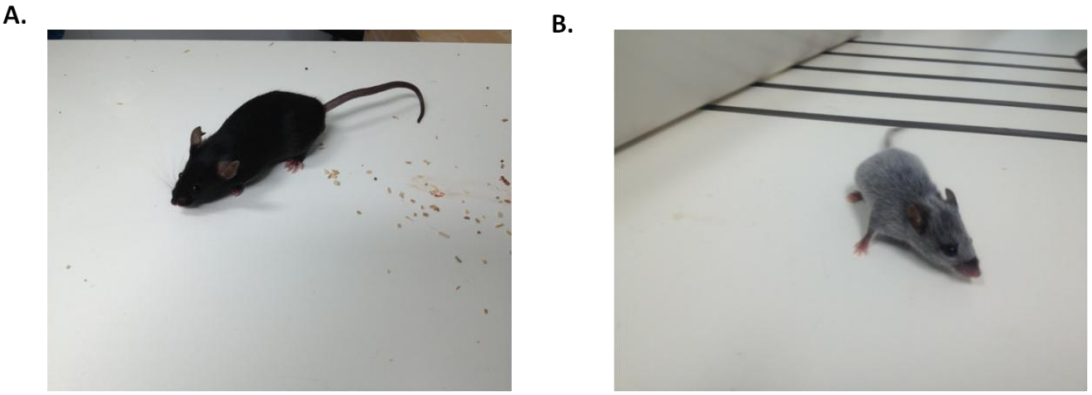


Fig. 2.1. Photographs of wild type and transgenic mice
(A) Wild type (WT); (B) Bcl-2 knockout (Bcl-2 KO). (Unpublished)

2. Preparation of solutions

1)Preparation of NaHEPES solution
NaHEPES solution was prepared as follows: 140 mM sodium chloride; 4.7 mM potassium chloride; 1.13 mM magnesium chloride; 10 mM HEPES, Free Acid; 10 mM D(+)glucose. PH was adjusted to 7.2 with NaOH. NaHEPES solution was supplemented with 1 mM calcium chloride for pancreatic acinar cell isolation and experiments.

2) Preparation of collagenase
20 ml of NaHEPES (supplemented with Ca^{2+}) buffer were added to 4000 u collagenase stock resulting in 200 u/ml collagenase solution. The solution was aliquoted into 20 x 1.5 ml tubes and stored at -20^{o}C.

3) Preparation of NMDG-HEPES solution
NMDG-HEPES solution was prepared as follows: 140 mM N-methyl-D-glucamine; 4.7 mM potassium chloride; 1.13 mM magnesium chloride; 10 mM HEPES; 10 mM D(+)glucose. PH was adjusted to 7.2 with HCl.

3. Isolation of pancreatic acinar cells

C57BL6/J mice were sacrificed by cervical dislocation in accordance with the Animal (Scientific Procedures) Act, 1986. The pancreas was removed from a mouse and washed twice with NaHEPES buffer with 1 mM Ca^{2+}. Then the tissue was injected with 1 ml 200 u/ml collagenase. After that, the tissue was collected into a 1.5 ml eppendorf tube and put into water bath for 15 min incubation with collagenase solution at 37^{o}C. When the incubation was finished, the tube was filled up with NaHEPES + 1 mM Ca^{2+}. The tissue suspension was pipetted up and down to release single cells and the supernatant was collected to a 15 ml tube. Fresh NaHEPES buffer (+ 1mM Ca^{2+}) was added again to the tube and the procedure of supernatant collection was repeated several times. Then, the cells were spun for 1 min at 1200 RPM in the centrifuge. The supernatant was discarded and the cell pellet was resuspended in 10 ml of NaHEPES buffer (+ 1 mM Ca^{2+}). The cell suspension was filtered through the filter mesh and spun again for 1 min. The final pellet was resuspended in 2 ml NaHEPES buffer (+ 1 mM Ca^{2+}).

4. Cell [Tissue] culture

AR42J cells (rat exocrine pancreatic tumour cells) were obtained from ECACC General Collection. AR42J cells were maintained in RPMI 1640 medium with 10 mM HEPES, 10 % FBS, 50 μg/ml gentamycin (GIBCO) and 2.5 μg/ml Fungizone; at 37^{o}C, 5 % CO2 in 25 cm^3 tissue culture flasks. Cell cultures were split once a week: trypsin was used to detach the cells and 1/10 of the total cell suspension was transferred to a new flask with 10 ml fresh medium. For the purpose of microscope visualization, cell

cultures were plated onto 5 cm^3 glass bottom dishes, allowed to attach for 2 days, then transfected with Bcl-2-GFP plasmid, ER CaM plasmid or siRNA and incubated for another 48 h.

5. Calcium measurements in intact cells

For ratiometric cytosolic calcium measurements, freshly isolated pancreatic acinar cells or AR42J cells were loaded with AM form of the calcium sensitive dye, fura-2, for 45 min at room temperature or for 1 h at 37^oC, respectively. The final concentration of the dye was 10 μM. After incubation, the dye was replaced with fresh NaHEPES buffer supplemented with 1 mM Ca^{2+}, and calcium changes in the cytosol were recorded using Nikon Diaphot 200 imaging system (excitation 340 nm and 380 nm, green emission with a peak at 510 nm; or with LED excitation 365 nm and 385 nm, green emission at 510 nm).

Freshly isolated pancreatic acinar cells were loaded calcium sensitive dye Fluo-4,AM for 30 min at room temperature, the final concentration of the dye was 5 μM. After incubation the dye was replaced with fresh NaHEPES buffer supplemented with 1 mM Ca^{2+} and cells were used for experiments on confocal system (excitation 488 nm; emission > 510 nm).

6. Calculations of cytosolic calcium extrusion rates

To calculate calcium extrusion rates in fura-2-loaded pancreatic acinar cells, fura-2 ratio obtained upon excitation with 340/380 nm light was converted into free calcium concentration using Grynkiewicz equation:
$[Ca^{2+}] = K_d \times \beta \times (R - Rmin) / (Rmax - R)$
Symbols:
K_d – dissociation constant of Ca^{2+} binding site, assumed 224 nM.
β – coefficient equal to the ratio of fluorescence emission intensity at 380 nm for Ca^{2+}-free and Ca^{2+}-bound fura-2.
R – ratio value
Rmin – minimal ratio value
Rmax – maximal ratio value
Coefficient β was calculated for known $[Ca^{2+}]$ values. Separate calibration experiments were performed in order to obtain extreme ratio values: Rmax was obtained by treatment of fura-2-loaded cells with 20 μM ionomycin; and Rmin – by perfusion with 2 mM EGTA for 30 min after permeabilization with ionomycin.

7. Cell death assay

Sigma Annexin V-FITC Apoptosis Detection Kit was applied for cell death assay on freshly isolated pancreatic acinar cells. After treatment, all the groups were incubated with 5 ml Annexin V-FITC at room temperature for 15 min. Subsequently, 10 ml

propidium iodide was added to all samples. Annexin V-FITC staining of apoptotic cells was visualized by Annexin V-FITC staining and they were excited by 488 nm laser light; propidium iodide staining of necrotic cells was detected when excited by 535 nm laser light. Multiple pictures were taken within in every group, and the live, apoptotic and necrotic cells were counted afterwards. Average values and standard errors were calculated and presented in the bar charts.

8. Equipment and software

Fura-2 recordings were performed on (1) Nikon Diaphot 200 imaging system using software Image-Pro Plus, MediaCybernetics; (2) and Photometrics – Cairn imaging system using software Image-Pro Plus, MediaCybernetics. Microsoft Word 2007 was the main tool for analyzing and editing this work. Calculations, graphs and charts were made in Microsoft Excel 2007. Cell images were taken from Leica confocal software. Diagrams were prepared in Microsoft PowerPoint and Adobe Photoshop Version 9.0. The reference list was generated using Reference Manager Professional Edition Version 10.

Chapter 3:

The role of Bcl-2 protein in regulation of calcium fluxes across the plasma membrane

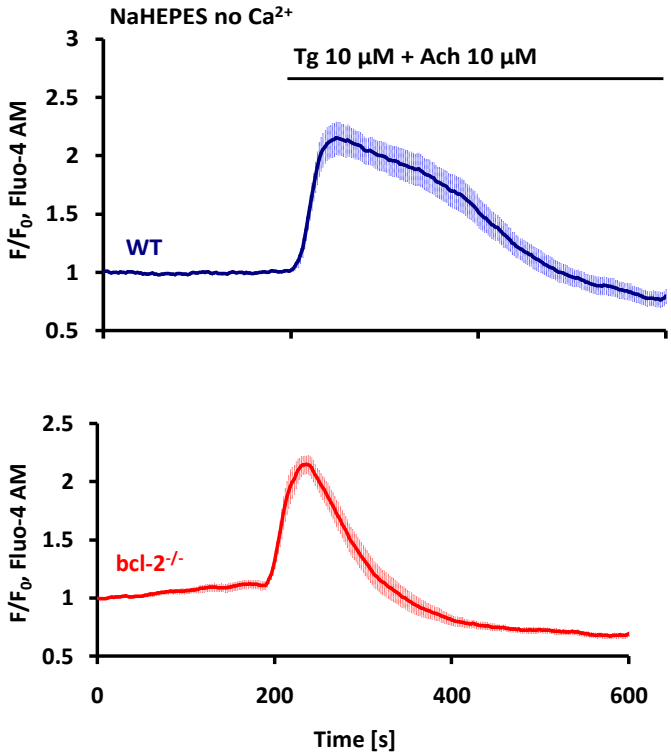
Chapter 3: The role of Bcl-2 protein in regulation of calcium fluxes across the plasma membrane

3.1. Loss of Bcl-2 affects calcium signalling in pancreatic acinar cells

In this chapter, the Bcl-2 protein is postulated to be a potential and novel regulator of calcium extrusion across the plasma membrane. The Bcl-2 family proteins were found not only to play an important role in regulating intracellular calcium homeostasis but also the preliminary data depicted in Fig. 3.1 indicated that Bcl-2 might affect the calcium extrusion across the plasma membrane in pancreatic acinar cells.

Pancreatic acinar cells were isolated from pancreas of wild type (WT) and Bcl-2 knock out (Bcl-2 KO) mice. The cells were subsequently loaded with calcium indicator Fluo-4, AM, which is cleaved inside the cell to the free and sensitive dye, fluorescent Fluo-4. Without calcium in external solution, the cells were perfused by combination with 10 μM ACh and 10 μM thapsigargin. Thapsigargin (Tg) is a specific inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which is a calcium pump located in the ER of the pancreatic acinar cells. It can utilize ATP hydrolysis for transferring Ca^{2+} from the cytosol to the lumen of the ER. Under this treatment, intracellular calcium stores were quickly emptied and Ca^{2+} could only be removed from the cytosol through the plasma membrane. Average responses in Fig.3.1 show that Ca^{2+} was extruded faster in Bcl-2 KO cells than WT cells. In order to compare Ca^{2+} responses, areas between 200 s and 400 s under the traces from Fig. 3.1.A were calculated. As the Fig. 3.2.B bar chart shows that cytosolic calcium responses in WT cells appear significantly larger than the responses in Bcl-2 KO pancreatic acinar cells ($p = 0.002$).

A.



B.

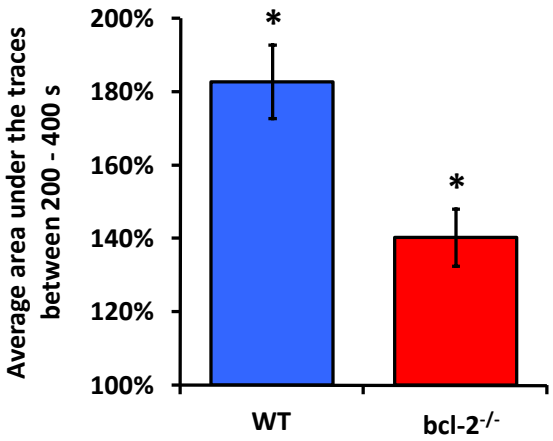


Fig. 3.1. Responses to thapsigargin and acetylcholine dramatically reduced in Bcl-2 knockouts.

(A) *Control traces (blue) demonstrate calcium responses to a mixture 10 µM acetylcholine and 10 µM thapsigargin in pancreatic acinar cells isolated from wild type (n=11). Red traces show calcium responses to a mixture 10 µM acetylcholine and 10 µM thapsigargin in fluo-4-loaded pancreatic acinar cells isolated from and Bcl-2 knockout mice (n=23). (Modified from Ferdek et al, 2012). [63]* ***(B)*** *Area under the curve has been calculated between 200 and 400 s and shows a significant reduction in traces from knockout mice (p=0.002). (Modified from Ferdek et al, 2012). [63]*

3.2. Bcl-2 regulates calcium extrusion across the plasma membrane in pancreatic acinar cells.

In order to investigate the calcium extrusion, we compared the different calcium signalling mechanisms in pancreatic cells isolated from wild-type (WT) and Bcl-2 knockout (Bcl-2 KO) mice. The calcium sensitive dye fura-2, AM was applied for fluorescent Ca^{2+} measurements in freshly isolated pancreatic acinar cells. Fura-2, AM is a cell permeable, high affinity, ratiometric and sensitive indicator dye for measuring intracellular calcium. Carboxylic acids are modified with acetoxymethyl (AM) ester groups, which results in uncharged molecules crossing cell membranes. Once fura-2, AM is inside the cell, AM groups are cleaved by nonspecific esterase. This leads molecules to be charged that makes them leak from the cell far more slowly than in their previous form. Fura-2 only has good sensitivity below the 1μM $[Ca^{2+}]_i$. For ratiometric measurement, fura-2 is excited at 340/380 nm and its fluorescent emission is recorded at 510nm. In these experiments, pancreatic acinar cells were loaded with fura-2, AM.

These cells were perfused by the NaHEPES solution without Ca^{2+} and treated by 2μM thapsigargin (Tg). Since the SERCA was blocked, calcium could not be pumped back to the ER, which caused the ER calcium stores to empty. After the depletion of the ER calcium stores and the $[Ca^{2+}]_i$ reached the baseline, 1mM, 5mM or 10mM extracellular Calcium were applied. The rapid calcium elevation was shown in these traces, as the extracellular calcium entered the cytosol via the store-operated Ca^{2+} (SOC) channels. When the stable cytosolic $[Ca^{2+}]_i$ plateau was reached, extracellular Ca^{2+} was removed. Because the SERCA was still blocked by Tg, calcium in the cytosol could only be extruded by the Ca^{2+} pump in the plasma membrane. Then the $[Ca^{2+}]_i$ declined to the resting level. (Fig.3.2. A). We compared the initial $[Ca^{2+}]_i$ between the WT and Bcl-2 KO cells. It is significant that the $[Ca^{2+}]_i$ of Bcl-2 KO cells (57.5 ± 3 nM SE) is lower than WT cells (100.3 ± 5.6 nM SE). This significant difference suggests that the change in equilibrium of calcium flux had occurred across the plasma membrane. (Fig.3.2. B)

A.

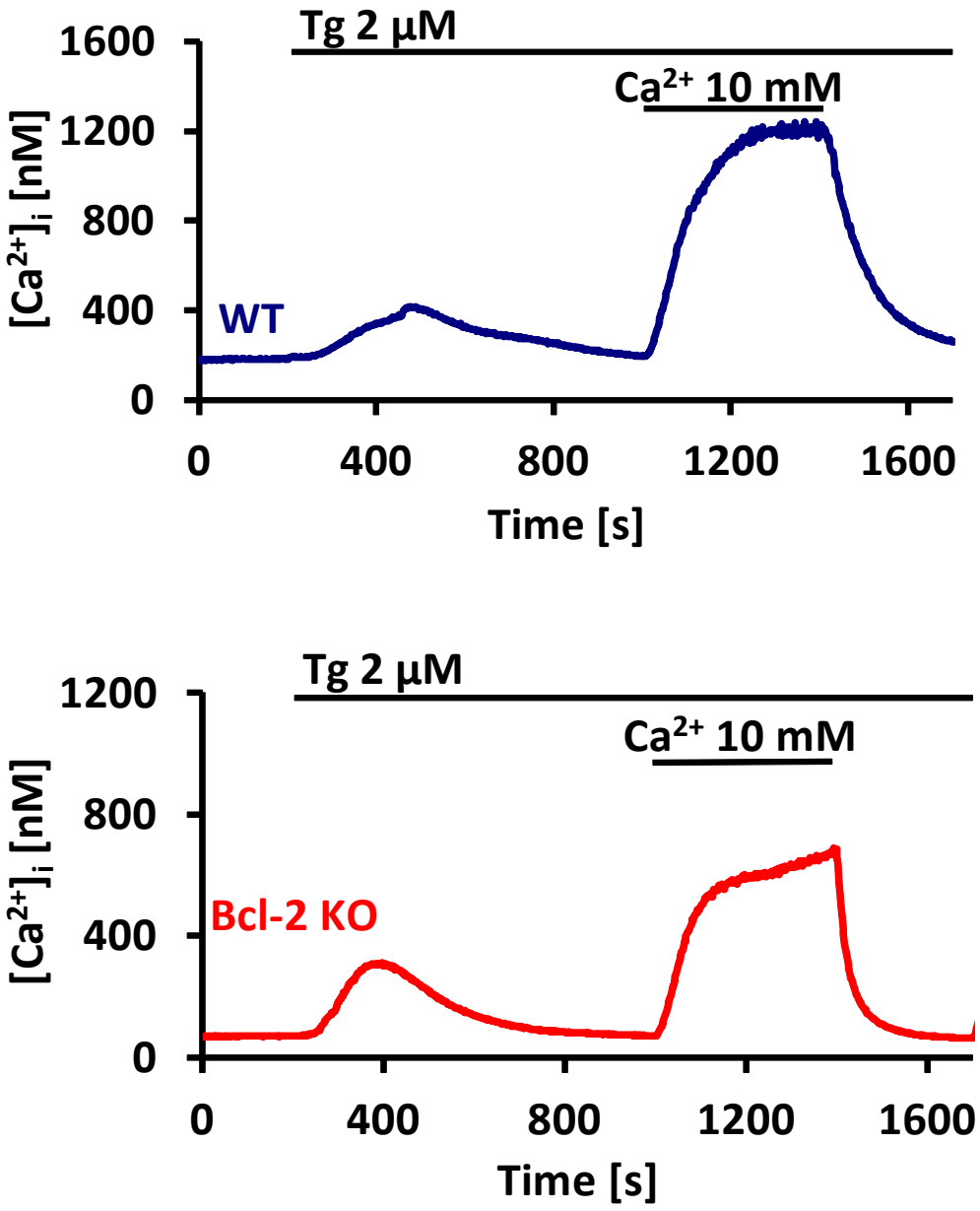


Fig 3.2. Bcl-2 regulates Ca^{2+} extrusion across the plasma membrane

(A) *Example traces recorded $[Ca^{2+}]_i$ response in WT and Bcl-2 KO pancreatic acinar cell. The cell exposed to the free calcium extracellular solution. After the application of 2µM thapsigargin (Tg), the intracellular stores were depleted. Thereafter, the elevation of $[Ca^{2+}]_i$ was caused by external 10 mM Ca^{2+}. The rate of reducing $[Ca^{2+}]_i$ was followed by removal of the high Ca^{2+} external solution. (Modified from Ferdek et al, 2012). [63]*

B.

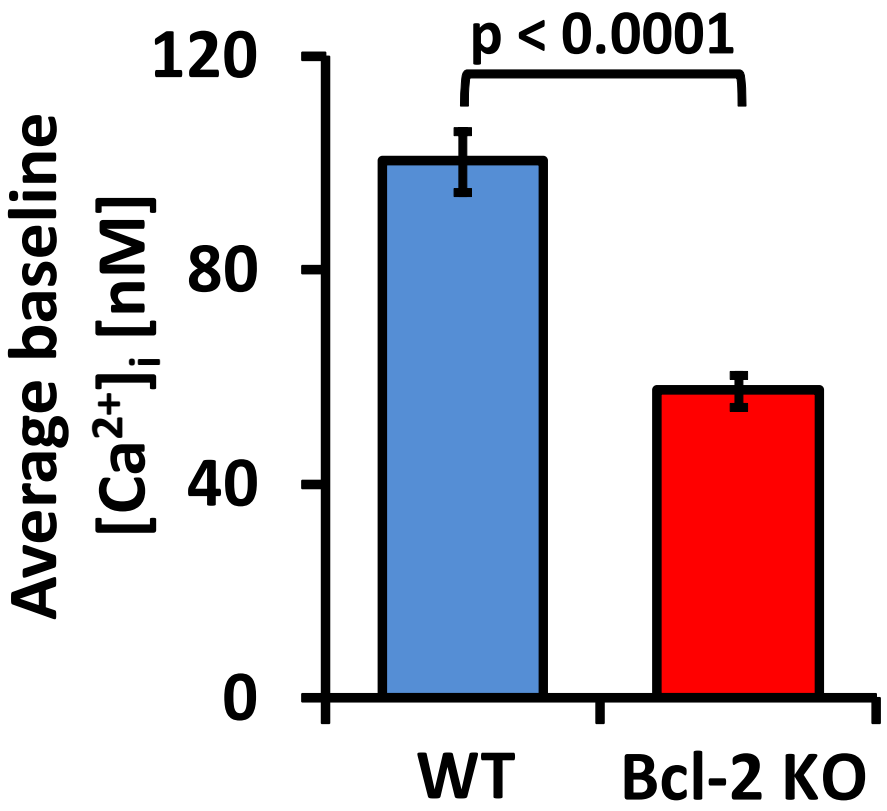


Fig 3.2. *(continues)*
(B) *Comparison of the resting $[Ca^{2+}]_i$ in WT (blue bar, n = 34) and Bcl-2 KO (red bar, n = 109) pancreatic acinar cells (p < 0.0001). (Modified from Ferdek et al, 2012). [63]*

3.3. Loss of Bcl-2 enhances Ca^{2+} extrusion across the plasma membrane

In order to fully understand the difference in calcium extrusion between WT and Bcl-2 KO pancreatic acinar cells, multiple experiments and mathematical methods were required to trace the Ca^{2+} changes in the cytosol of the cells. Using the same protocol as the previous experiment, WT and Bcl-2 pancreatic acinar cells were loaded with fura-2, AM dye. The cells were perfused with 2μM Tg with Ca^{2+} free solution at first. Because the SERCA was blocked by Tg, Ca^{2+} could not be taken up into the ER. When the $[Ca^{2+}]_i$ returned to the basal levels, the extracellular solution with Ca^{2+} was rapidly changed to 1mM, 5mM or 10mM. After the $[Ca^{2+}]_i$ reached the plateau, the extracellular calcium was removed. As a result $[Ca^{2+}]_i$ returned to the baseline level.

All the traces recorded in these experiments with WT and Bcl-2 KO pancreatic acinar cells are shown in Fig. 3.2. A and B (nWT = 45, nBcl-2 KO= 109). The recovery phase of the high calcium transient from every trace was calculated and then plotted as a function of the $[Ca^{2+}]_i$ values obtained from the exponential fit. The result clearly demonstrates that calcium extrusion in Bcl-2 KO cells was much faster than WT cells. The half-time of recovery of baseline $[Ca^{2+}]_i$ levels was also calculated; the result shows that the half-times ($\tau 1/2$) of the reduction in $[Ca^{2+}]_i$ toward the resting level following removal of external Ca^{2+} in WT cells (n = 20) was much slower than Bcl-2 KO cells (n = 38)) shown in Fig. 3.3 .

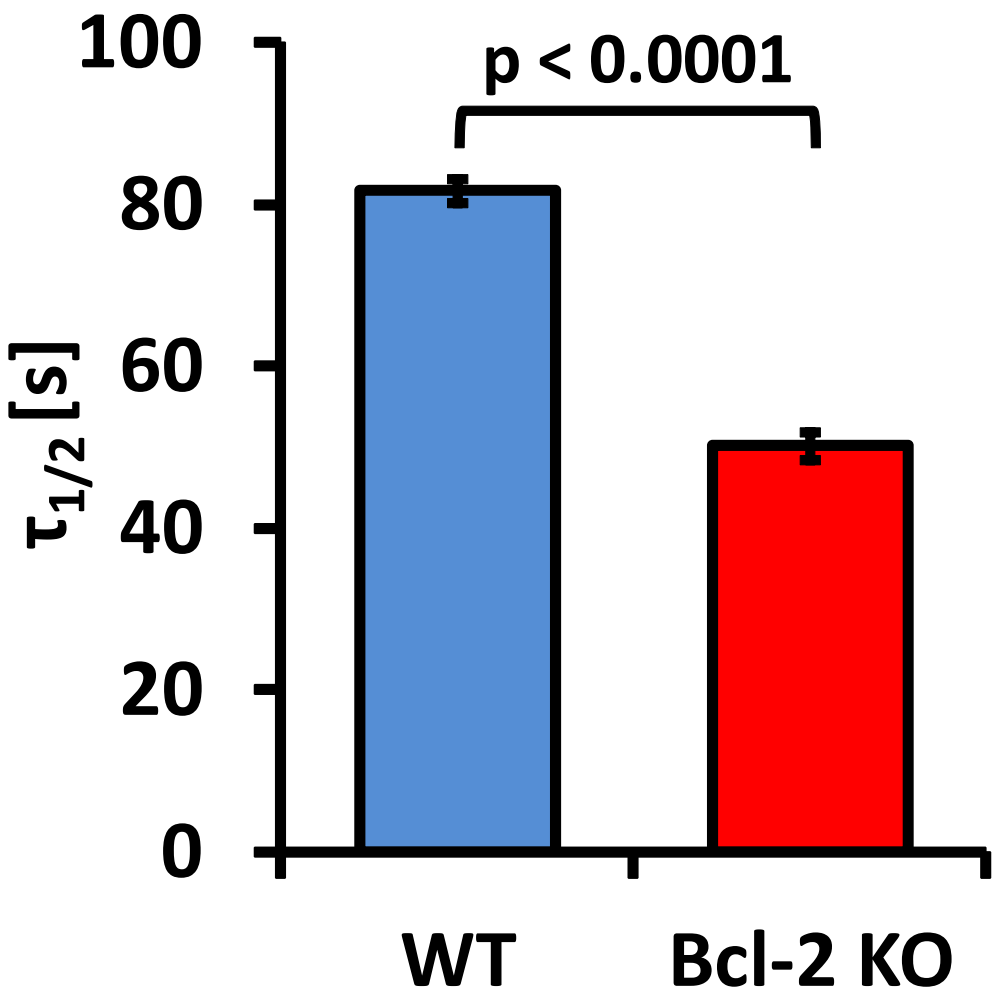


Fig. 3.3. Loss of Bcl-2 increases Ca^{2+} clearance from the cytosol
Bar chart shows that the half-time (τ1/2) of the [Ca^{2+}] recovered to the resting level following removal of external Ca^{2+} in WT (blue bar, n = 20) and Bcl-2 KO (red bar, n = 38) cells. (Modified from Ferdek et al, 2012). [63]

3.4. Cytosolic calcium extrusion in pancreatic acinar cells is mainly dependent on the plasma membrane calcium-activated ATPase rather than the sodium-calcium exchanger

Generally, cytosolic calcium is extruded across the plasma membrane in two ways: 1) the plasma membrane calcium-activated ATPase (PMCA) [64] and the Na^+/ Ca^{2+} exchanger (NCX) [65]. The PMCA is the vital regulator of $[Ca^{2+}]_i$; it is a transport protein in the plasma membrane of cells powered by the hydrolysis of adenosine triphosphate (ATP). It removes one Ca^{2+} ion for each ATP hydrolysed. The PMCA has a high affinity to calcium ions, but its action in removing calcium from the cytosol to the extracellular space is rather slow. Thus, the PMCA is effective at resting maintaining $[Ca^{2+}]_i$ at its physiologic levels. Because of this function of the PMCA, calcium as the important second messenger can maintain the accurate signalling within pancreatic acinar cells [66].

In contrast the NCX is an antiporter membrane protein that has a low affinity to Ca^{2+}, but it can use the electrochemical gradient of sodium, transporting large amounts of Ca^{2+} quickly from the cells. Therefore its capacity depends on the concentration of Ca^{2+} inside the cells. The NCX helps to recover the physiological $[Ca^{2+}]_i$ by removing substantial Ca^{2+} from the cytosol in a short time. A single Ca^{2+} is removed by NCX in exchange for the import of three Na^+ [67]. It was established however that the NCX does not play the most important role in Ca^{2+} extrusion from pancreatic acinar cells [68]. The PMCA is the main regulator of transporting calcium across the plasma membrane and it may indeed be the target of Bcl-2 from the data showed before.

In order to test if the NCX plays any significant role in Ca^{2+} extrusion and how Bcl-2 may regulate this we designed two different experiments. In the first experiment, we compared in Bcl-2 KO cells and normal pancreatic acinar cells the half-times ($\tau 1/2$) of the reduction of $[Ca^{2+}]_i$ toward the resting level following removal of external Ca^{2+}. In order to indirectly inhibit the NCX, we substituted Na^+ with N-methyl D-glucamine ($NMDG^+$) as until now there has been no specific inhibitor of the NCX and this transporter's function is determined by the presence of extracellular Na^+.

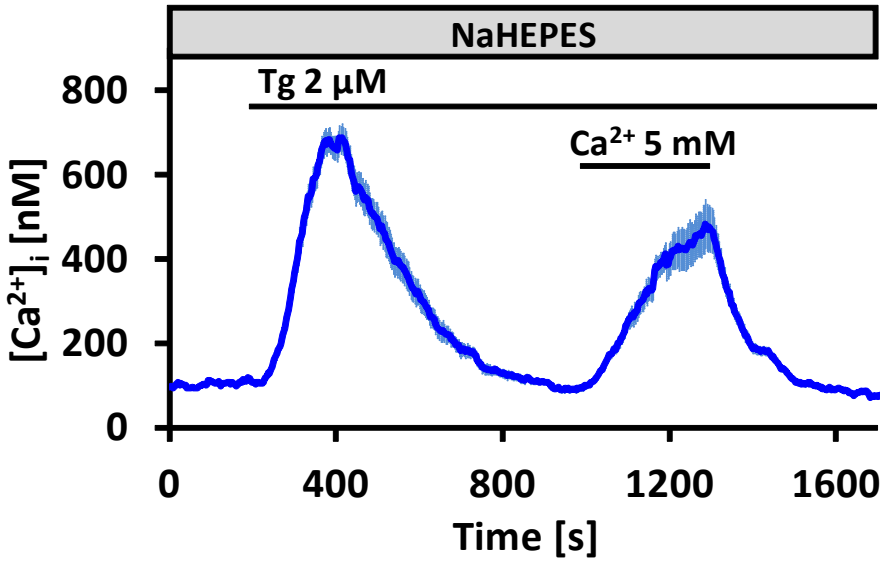
The WT cells and Bcl-2 KO cells were loaded with the fura-2, AM. The cells were exposed to the Ca^{2+} free NaHEPES solution. Following treatment with Tg 2μM the SERCA was blocked, emptying the ER store of calcium. After the Tg-induced cytosolic Ca^{2+} elevation had recovered to the basal $[Ca^{2+}]_i$, the extracellular NaHEPES solution was replaced by NMDG-HEPES with no Ca^{2+}. This was followed by another perfusion with Tg 2μM. Then, 5mM Ca^{2+} was applied to the extracellular solution. This induced the rapid influx of Ca2+ to the cytosol. When the Ca^{2+} elevation achieved its plateau, Ca^{2+} was removed from the NMDG-HEPES solution. This resulted in cytosolic Ca^{2+} clearance across the plasma membrane. In WT pancreatic acinar cells, the average control trace (n = 18) and average trace of Na^+ substitution with $NMDG^+$ (n = 24) are shown in the Fig. 3.4.A and B.

We collected all the traces from WT cells and Bcl-2 KO cells and calculated the average half-time of returning to the basal $[Ca^{2+}]_i$. Fig. 3.4.C shows that there was no difference from the control in the half-time of recovery to the resting $[Ca^{2+}]_i$ in the WT control cells with $NMDG^+$ replacement. There was also no difference in the Bcl-2 KO cells. However the Ca^{2+} extrusion is much faster in the Bcl-2 KO groups than the WT groups when comparing the WT controls and Bcl-2 controls as well as the WT with $NMDG^+$ substitution and Bcl-2 with $NMDG^+$ substitution. These results confirm that the NCX does not play the main role in cytosolic Ca^{2+} clearance in pancreatic acinar cells. The effects of Bcl-2 on Ca^{2+} extrusion must therefore be associated with PMCA functions.

In order to further investigate how Bcl-2 regulates Ca^{2+} extrusion, we designed a second experiment. Human Bcl-2 was transfected to the pancreatic cancer cell line AR42J and stably overexpressed. A similar protocol to the first experiment was applied in this experiment. WT AR42J cells and AR42J cells overexpressing Bcl-2 were loaded with fura-2, AM. At the beginning, cells were exposed to the NaHEPES solution without Ca^{2+}. Then, the cells were treated with 10μM cyclopiazonic acid (CPA), which is a fungal toxin and a specific inhibitor that similarly to thapsigargin, blocks the SERCA. Because CPA causes inhibition of store replenishment, after the application of the CPA, the calcium in the ER was depleted. When the $[Ca^{2+}]_i$ reached the resting level, external solution NaHEPES was substituted for NMDG-HEPES. After a 50 seconds perfusion with NMDG-HEPES, the cells were exposed to the high (10mM) extracellular Ca^{2+}. This induced the rapid and huge elevation of $[Ca^{2+}]_i$. When the plateau of $[Ca^{2+}]_i$ was obtained, the extracellular Ca^{2+} was removed.

We summarized the decline phase from AR42J control traces and AR42J overexpressing Bcl-2 traces and calculated the half times (τ1/2) of the $[Ca^{2+}]_i$ decrease towards resting levels following removal of external Ca^{2+}. Comparing the half-times of $[Ca^{2+}]_i$ recovery in Fig. 3.5.B we found that the overexpression of Bcl-2 (purple bar, n=127) slowed down Ca^{2+} extrusion in AR42J Cells (blue bar, n=44).

A.



B.

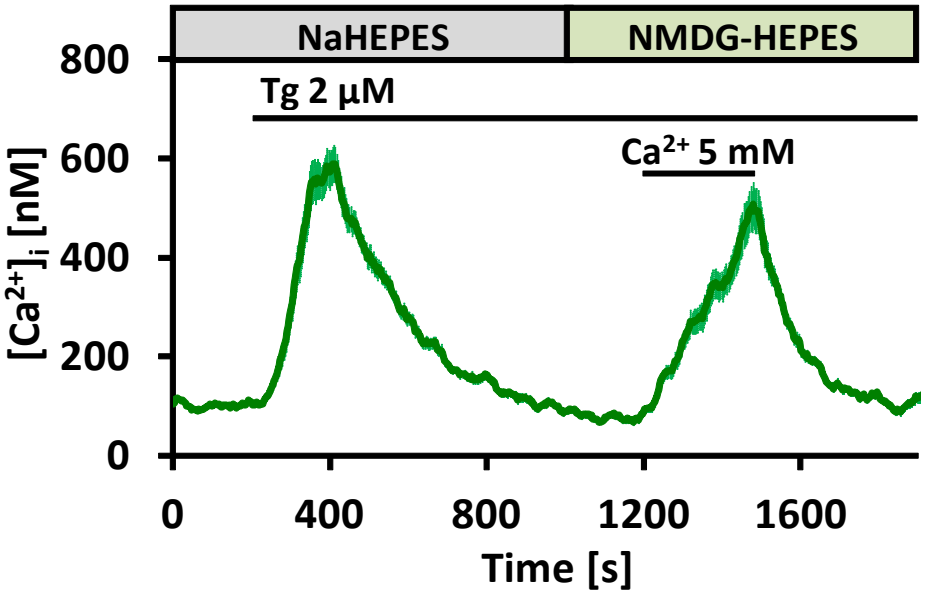


Fig. 3.4. Involvement of PMCA and NCX in Ca^{2+} extrusion in acinar cells

(A) *An average trace recorded $[Ca^{2+}]_i$ response in WT pancreatic acinar cells. After intracellular store depletion with 2 µM thapsigargin, the extracellular solution kept in NaHEPES buffer (n = 18; bars are standard errors). (Modified from Ferdek et al, 2012). [63]*

(B) *An average trace recorded $[Ca^{2+}]_i$ response in WT pancreatic acinar cells. The same protocol was similar to (A). However, the extracellular Na^+ was substituted by $NMDG^+$ in order to inhibit NCX (n = 24; bars are standard errors). (Modified from Ferdek et al, 2012). [63]*

C.

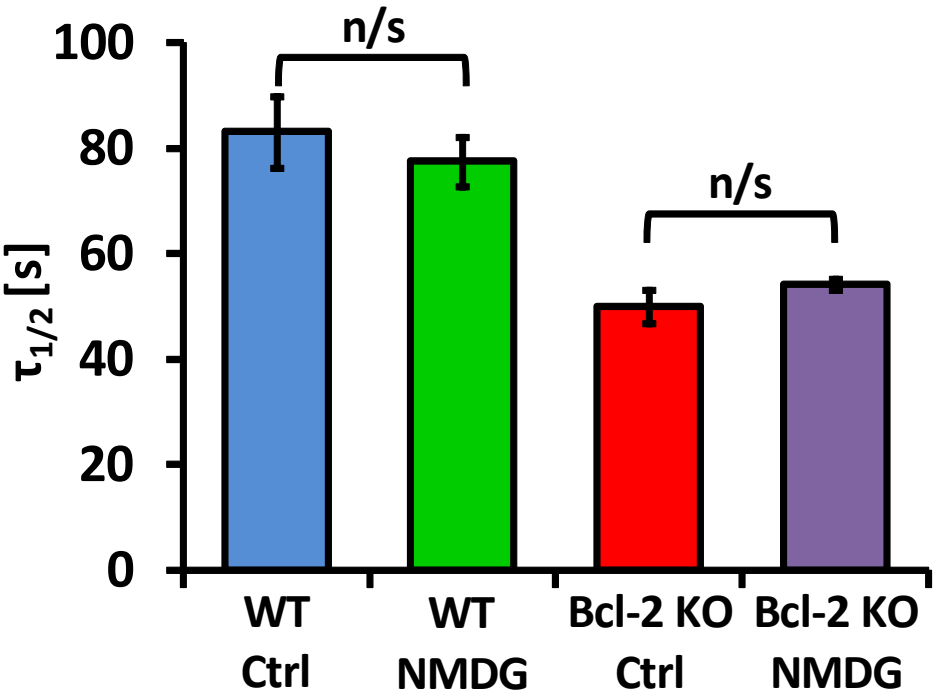


Fig. 3.4 (continues)

(C) *Bar chart shows the comparison of half-times (τ1/2) of $[Ca^{2+}]_i$ recovered toward the resting level following removal of external Ca^{2+} (blue bar, n = 18) or when external Na^+ was replaced by $NMDG^+$ (green bar, n = 24) in WT pancreatic acinar cells as well as in Bcl-2 KO pancreatic acinar cells (red bar, in the presence of Na^+, n = 6; purple bar, when Na^+ was replaced by $NMDG^+$, n = 25). (Modified from Ferdek et al, 2012). [63]*

A.

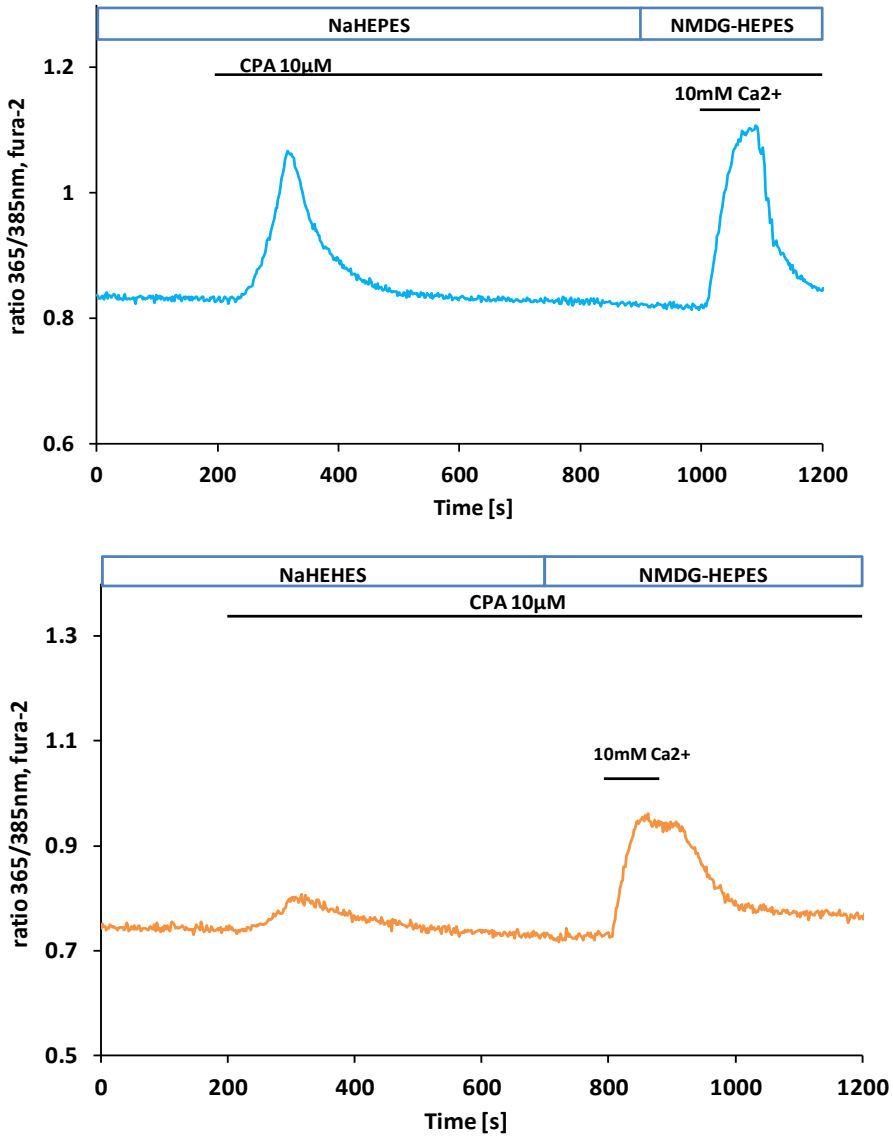


Fig. 3.5. Overexpression of Bcl-2 in AR42J Cells Decreases PMCA-dependent Cytosolic Ca^{2+} Extrusion across the Plasma Membrane

(A) *Example traces show the changes in $[Ca^{2+}]_i$ in a Fura-2-loaded control AR42J cell and an AR42J cell overexpressing Bcl-2. Changes in $[Ca^{2+}]_i$ were evoked first by application of 10µM cyclopiasonic acid (CPA), which depleted the ER store. Then, extracellular Na^+ was substituted for $NMDG^+$ in the external solution with 50s briefly perfusion before the cell was exposed to high (10mM) extracellular Ca^{2+}. After removal of 10mM Ca^{2+} from the external solution, the $[Ca^{2+}]_i$ reduced to the resting level. (Original traces used for Figures S3C,D in: Pawel E. Ferdek, Julia V. Gerasimenko, Shuang Peng et al, 2012). [63]*

B.

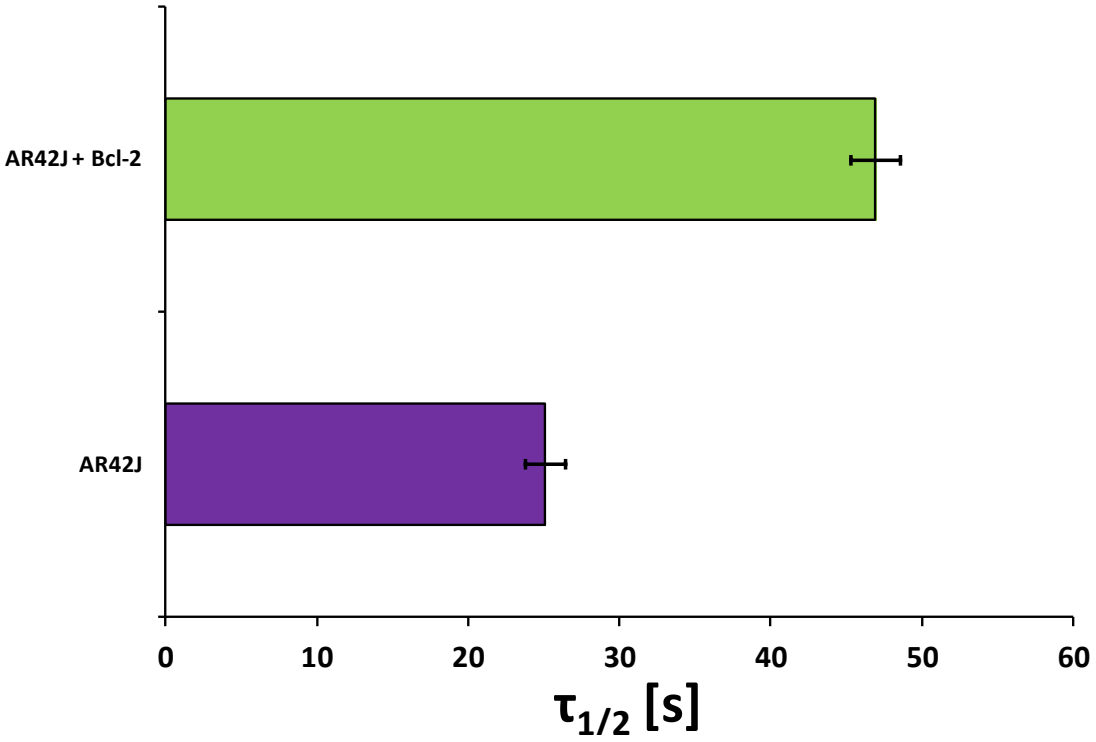


Fig. 3.5. *(continues)*
(B) *The bar chart shows the comparison of half-times (τ1/2) of* $[Ca^{2+}]_i$ *reduced to the resting level following removal of external* Ca^{2+} *in Fura-2-loaded control AR42J cells (purple bar, n=44) and in AR42J cells overexpressing Bcl-2 (green bar, n=127). (Original data used to produce Figures S3E in: Pawel E. Ferdek, Julia V. Gerasimenko, Shuang Peng et al, 2012). [63]*

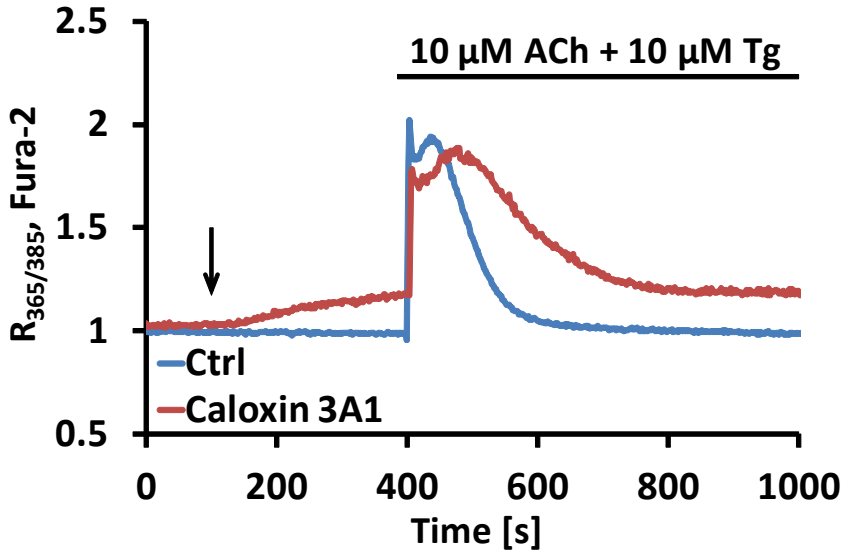
3.5. Inhibition of PMCA by Caloxin 3A1 Promotes Necrosis

In order to investigate the physiological importance of PMCA-mediated Ca^{2+} extrusion, we used the specific PMCA inhibitor peptide caloxin 3A1 [69]. In the control experiment, freshly isolated WT pancreatic acinar cells were loaded with fura-2, AM. Then, application of 10μM ACh and 10μM Tg induced $[Ca^{2+}]_i$ elevation and emptied the ER store. In the contrast experiment, the pancreatic acinar cells were preincubated with 1mM caloxin 3A1 for 5 minutes. After 100 seconds perfusion with NaHEPES with 1mM Ca^{2+}, caloxin 3A1 was added to the external solution. This led to a sudden increase in the $[Ca^{2+}]_i$. Then the same protocol as in the control experiment was applied. Fig. 3.6.A shows the $[Ca^{2+}]_i$ change between the untreated pancreatic acinar cells and the pancreatic acinar cells with caloxin 3A1 and indicates that caloxin 3A1 increased the basal resting $[Ca^{2+}]_i$ effectively. We compared the half-time of the $[Ca^{2+}]_i$ recovery to the basal level in Fig. 3.6.B; this result demonstrates that caloxin 3A1 significantly inhibits Ca^{2+} extrusion by PMCA and doubles the recovery time.

In order to investigate the pathophysiological consequences of inhibition of PMCA-mediated Ca^{2+} extrusion, we performed cell death assays on WT pancreatic acinar cells treated with 30mM menadione [8] and/ or 1mM Caloxin 3A1 [69] for 30 min. Menadione is a quinone that is metabolised by flavoprotein reductase to semiquinone, which can be oxidised back to quinone in the presence of molecular oxygen. In this redox cycle the superoxide anion radical, hydrogen peroxide and other reactive oxygen species are generated. Menadione can cause elevations in the cytosolic Ca^{2+} concentration contributing to cell death.

Freshly isolated pancreatic acinar cells were divided into four groups: control, caloxin 3A1, menadione alone and caloxin 3A1 with menadione. After 30 min loading of every group, propidium iodide was added to the cells for the necrosis assay. As the Fig. 3.6.C bar chart shows, there is no difference between the control and caloxin 3A1 group, which suggest that caloxin 3A1 itself cannot cause necrosis in pancreatic acinar cells. Comparing the menadione and caloxin group with the menadione alone group, we observed that there were more necrotic cells in the caloxin 3A1 with menadione group than in the menadione alone group.

A.



B.

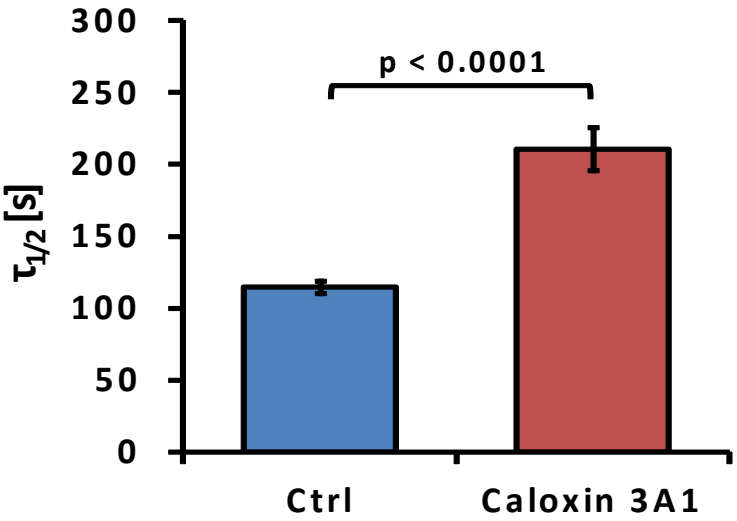


Fig. 3.6. Specific Inhibition of PMCA Substantially Increases Necrosis in Pancreatic Acinar Cells

(A) *Example traces recorded $[Ca^{2+}]_i$ response to 10 µM Tg and 10 µM acetylcholine (ACh) in an untreated pancreatic WT pancreatic acinar cell (blue trace) or a WT cell was treated with 1 mM caloxin 3A1 (dark red trace). Black arrow indicates time of caloxin 3A1 application. (Modified from Ferdek et al, 2012). [63]*

(B) *The bar chart shows the comparison of half-times (τ1/2) of $[Ca^{2+}]_i$ reduced to the resting level after the application of 10 µM Tg and 10 µM ACh in the presence (dark red bar, n = 26) or absence (blue bar, n = 15) of 1mM caloxin 3A1. (Modified from Ferdek et al, 2012). [63]*

C.

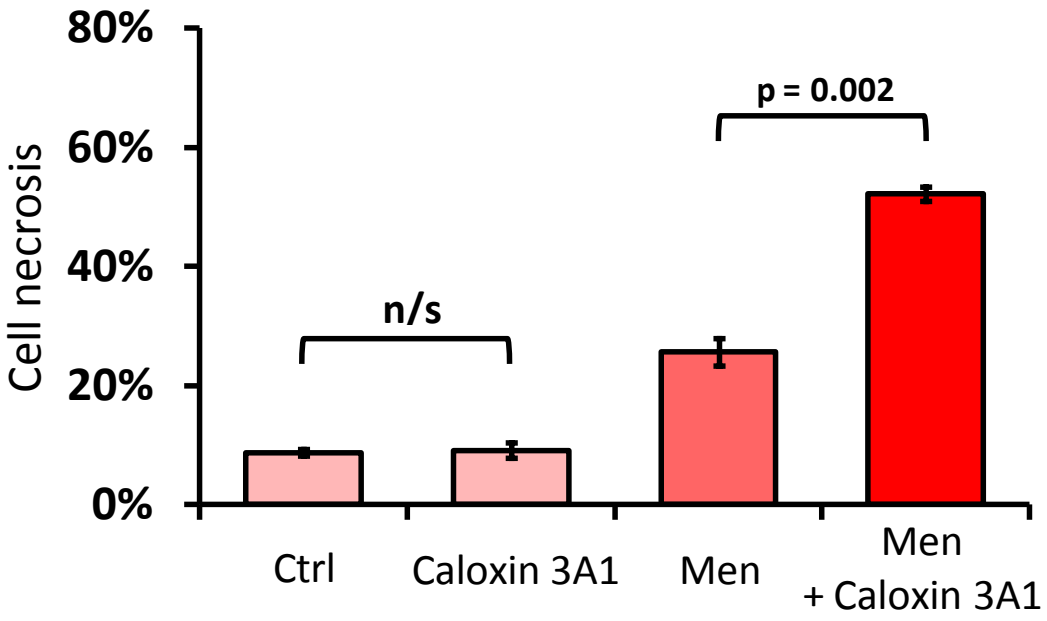


Fig. 3.6. *(continues)*

(C) *Bar chart shows the comparison of necrosis levels in WT pancreatic acinar cells under different conditions. Cells were incubated with 1mM caloxin 3A1 (n = 498), 30μM menadione (n = 485) , or 1 mM caloxin 3A1 and 30 μM menadione (n = 533) respectively for 30 minutes. In control (n = 521) , the cells without any treatment were incubated the same amount time. (Modified from Ferdek et al, 2012). [63]*

We conclude here that Bcl-2 plays a pathological role in pancreatic acinar cells through inhibition of PMCA and later in this dissertation we will discuss its role in comparison to that of the common calcium regulator calmodulin.

Chapter 4:

Protective role of calmodulin against necrosis in pancreatic acinar cells

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4.1. CALPs do not affect ACh-related physiological Ca^{2+} responses in pancreatic acinar cells

Calcium-like peptides (CALPs) are cell-permeable calmodulin (CaM) activators that bind to the EF-hand Ca^{2+}-binding site [70]. They were also shown to bind to cytoplasmic sites on certain Ca^{2+} channels, inhibiting Ca^{2+}-mediated cytotoxicity and apoptosis [71]. CALPs were designed by inversion of the hydrophobic pattern of the EF-hands. These peptides interact with Ca^{2+} binding motifs because they have a complementary surface contour to a stretch of eight amino acids of the loop sequence of EF hands [70]. The biological activity of the two such peptides, CALP1 and CALP3, was demonstrated by Manion et al [70]. They revealed that both of these octameric peptides were able to bind to the EF hands of CaM. The peptide/CaM complex in turn was able to activate phosphodiesterase in the absence of Ca^{2+}. This indicated the possibility that these peptides might block Ca^{2+}- and CaM-regulated Ca^{2+} channels.

As mentioned before, ACh and CCK are the most important physiological stimuli of pancreatic acinar cells. Small doses of ACh and CCK can cause cytosolic Ca^{2+} oscillation, which is essential for their secretory function. In order to investigate the effect of pre-activation of calmodulin with CALPs on calcium signalling in pancreatic acinar cells, several experiments were performed. Pancreatic acinar cells were isolated from wild type C57BL6 mouse pancreas; subsequently cells were incubated with fura-2, AM. In these experiments, CALPs were preincubated for 200s and after 200s cells were treated with physiological concentration of ACh 50 nM (Fig. 4.1). Normally 50 nM ACh induces global cytosolic Ca^{2+} oscillations, which originate in the granular pole of pancreatic acinar cells and spread towards the basolateral area. In all cases pre-treatment with CALP-1 (n = 12) or CALP-3 (n = 10) did not block ACh-induced oscillations.

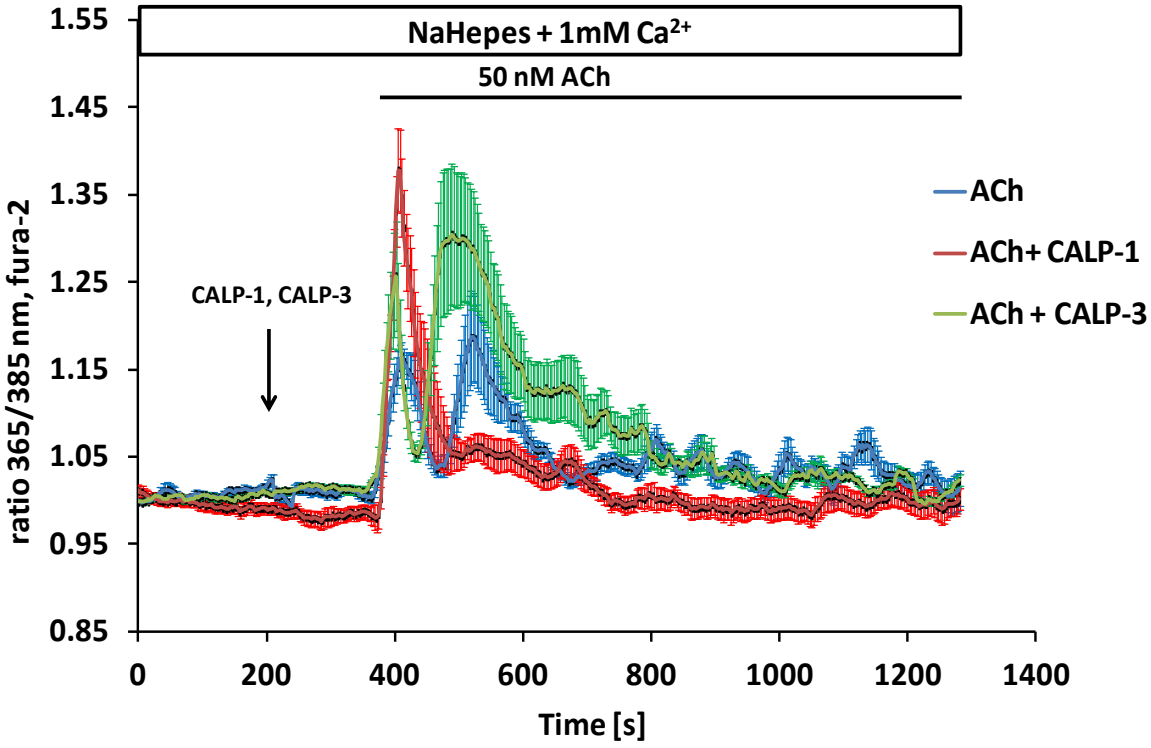
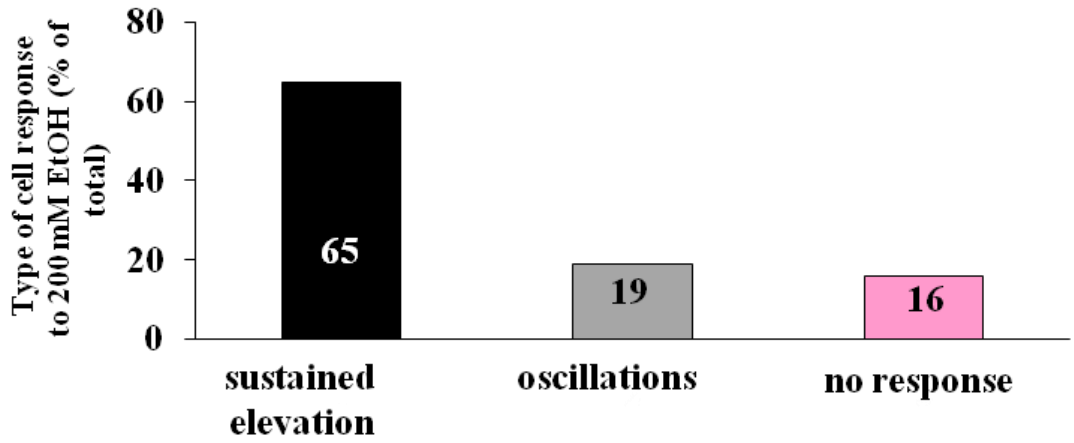


Fig. 4.1. CALPs do not significantly change ACh-induced Ca^{2+} responses
The three average traces obtained upon treatment of pancreatic acinar cells with: 50 nM ACh and 50 nM ACh before preincubation with 100 μ M CALP-1 or 100 μM CALP-3. (N>9 in each series). (Unpublished data).

4.2. Effects of CALP-3 on ethanol-induced Ca^{2+} responses

CALP-3 was demonstrated by Gerasimenko et al [72] to be effective in abolishing ethanol induced aberrant calcium signalling in pancreatic acinar cells. In this study, 200 mM ethanol was applied to wild type pancreatic acinar cells loaded with Fluo-4. In the control group, the majority of cells (65%, 28 cells of 43) responded with a small, sustained increase in $[Ca^{2+}]_i$ and a small portion of cells responded with delayed oscillatory $[Ca^{2+}]_i$ elevations (19%, 8 of 43 cells), whereas a smaller population (16%, 7 of 43 cells) did not show any response (Fig. 4.2.A). When cells were treated with CaM activator, the ethanol-induced $[Ca^{2+}]_i$ elevation was almost abolished by CALP-3. In the presence of 50 μM and 100 μM CALP-3, the percentage of cells responding to ethanol with a small sustained $[Ca2^{+}]_i$ elevation was reduced from 65% (Fig. 4.2.A) to 11% (Fig. 4.2.B, n = 18) and 0 (Fig. 4.2.C, n = 22), respectively. In other words, 100 μM CALP-3 could completely prevent ethanol-induced Ca^{2+} sustained responses. However, 23% of the cells still responded with small amplitude oscillations (to 0.12 + 0.05 ΔF/F0, Fig. 4.2.C).

A.



B.

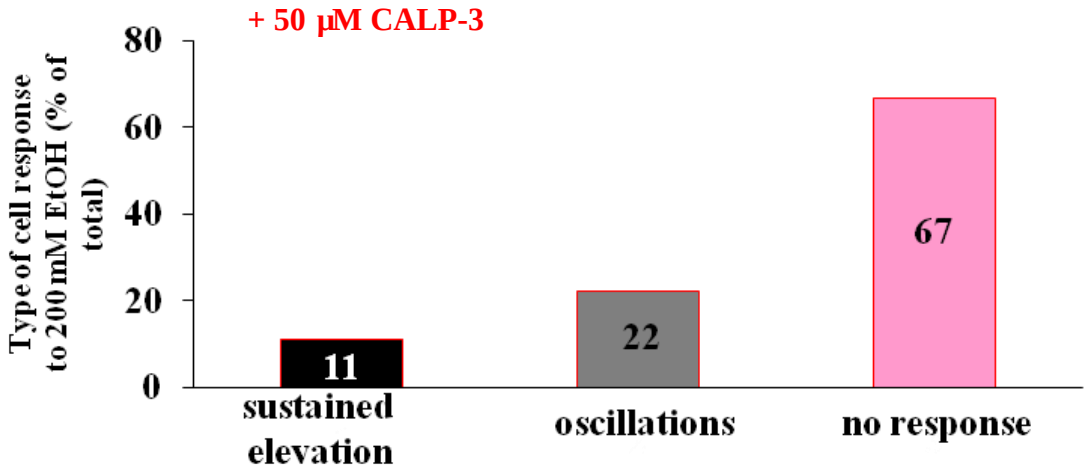


Fig. 4.2. Effects of CALP-3 on ethanol-induced Ca^{2+} responses in pancreatic acinar cells

In most cases, 200 mM ethanol causes small sustained elevations in $[Ca^{2+}]_i$ and some broad oscillations in intact pancreatic acinar cells. However, cells do not respond to the high concentration ethanol in some cases. The calmodulin activator CALP-3 effectively inhibits ethanol-induced calcium responses. (A) Percentage of cells responding to 200 mM ethanol either with a small sustained $[Ca^{2+}]_i$ elevation, or with broad oscillations, or not responding at all. (B) Percentage of cells responding to 200 mM ethanol with either a small sustained elevation, or short oscillations, or not responding at all in the presence of 50 µM CALP-3. (Modified from Gerasimenko et al. 2011). [72]

C.

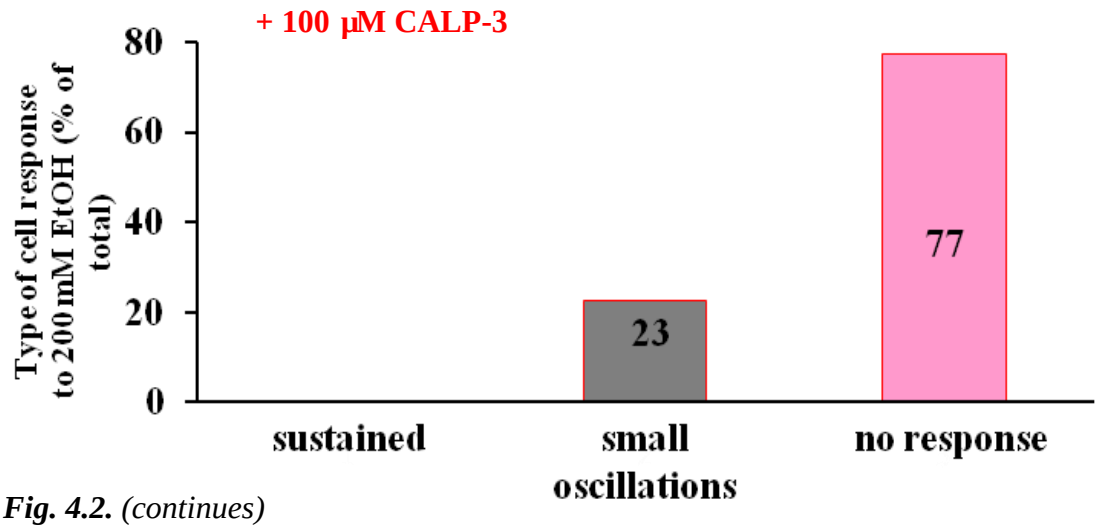


Fig. 4.2. *(continues)*

(C) Percentage of cells responding to 200 mM ethanol with either very small oscillations or no response in the presence of 100 µM CALP-3. (Modified from Gerasimenko et al. 2011). [72]

4.3. Activation of calmodulin by CALP-1 in intact cells protects against Menadione-induced necrosis

In order to investigate the protective role of CLAP-1 in cell death, menadione, an inducer of cell death, was applied in this experiment. As mentioned before, menadione can cause oxidative stress and elevations in the cytosolic Ca^{2+} concentration which contribute to cell death (Fig. 4.3.A). Cell death assays were performed with four samples on freshly isolated WT pancreatic acinar cells. Cells were pre-treated with 100 µM CALP-1 for 15 min or 30 µM Menadione for 30min at room temperature. After 15 min the samples with CALP-1 were incubated with Menadione for 30 min. Subsequently 5 µL Annexin-V-FITC was added to all samples and then incubated again for 15 min. At the end of the incubation, 10 µL propidium iodide was added. Control groups were incubated for 30 min without any treatment. Apoptotic and necrotic cells were visualized with Leica confocal microscope (Fig. 4.3.B). The combined results of the experiments are shown in Fig. 4.3.C. It is clear that CALP-1 can protect pancreatic acinar cells against necrosis ($P < 0.001$). The necrotic cells decreased to almost the same level as the controls, while the amount of apoptotic cells increased ($P = 0.004$).

A.

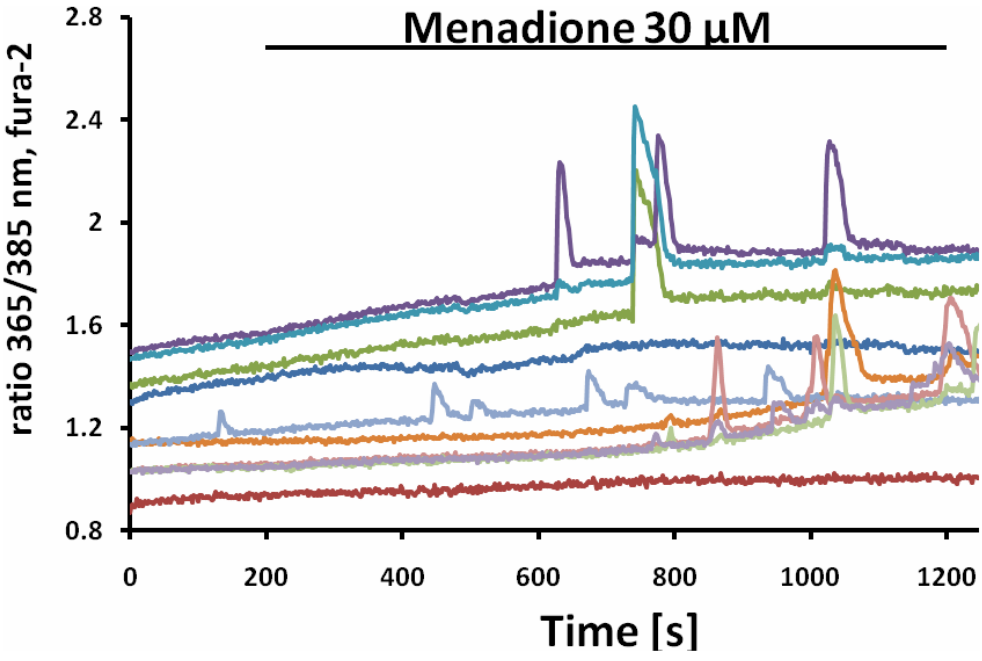


Fig. 4.3. A. Calcium responses induced by menadione
In freshly isolated pancreatic acinar cells loaded with fura-2, AM, 30 µM menadione induces cytosolic Ca^{2+} oscillations and sustained Ca^{2+} elevation (n = 9). (Unpublished data)

B.

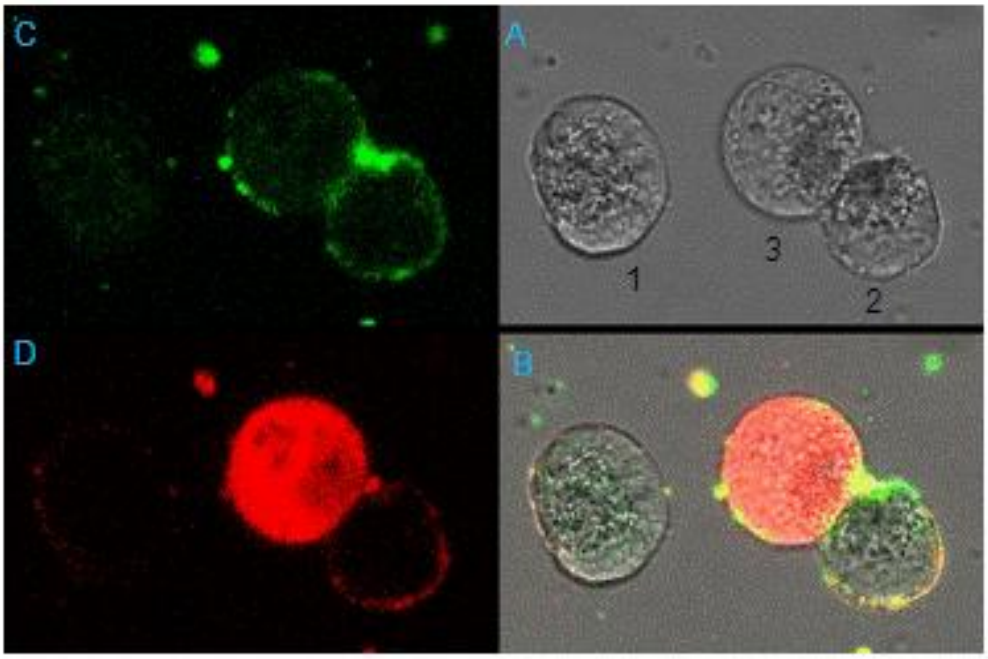


Fig. 4.3.B. Cell death assay checked by Apoptosis Detection Kit (Sigma).
The confocal images show the surviving and dead cells. (A) Transmitted light image shows pancreatic acinar cells. (B) Fluorescent image, overlay images of (A), (C) and (D), show a live cell and two dead cells. No.1 is a live cell without any staining. No.2 is apoptotic cell, which is only with green ring Annexin staining in the membrane. No.3 is necrotic cell, which is not only with green ring Annexin staining in the membrane, but also with red propidium iodide staining inside the cell. (Unpublished data)

C.

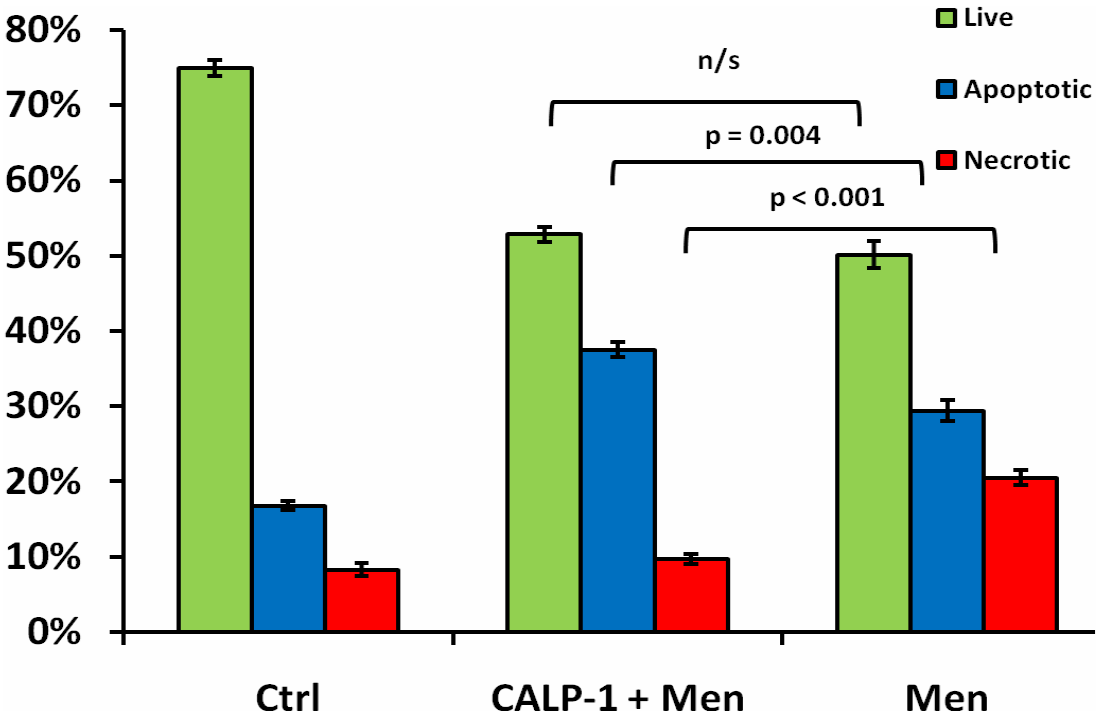


Fig. 4.3.C. CALP-1 promotes apoptosis and protects against necrosis
Results of cell death assays performed on WT pancreatic acinar cells. Light green bars represent live cells, blue bars represent apoptotic cells, and red bars represent necrotic cells. The chart is separated into three treatment groups, from the left: untreated control cells (n = 418), cells treated with both 30 μM menadione and 100 μm CALP-1 (n = 277), cells only treated with 30 μM menadione (n = 402). CALP-1 protects cells against necrosis and switches it to apoptosis when cells were challenged with 30 μM menadione. (Unpublished data)

Chapter 5:

Discussion

Chapter 5: Discussion

Bcl-2 family proteins and calcium modulated protein calmodulin share one common feature – they both regulate calcium signalling system. Since Ca^{2+} is an important second messenger within the cytoplasm, its distribution must be tightly regulated in different ways for transport, buffering and storage. Bcl-2 family proteins are well known as the crucial regulators of apoptosis. Currently it is widely accepted that Bcl-2 proteins are unevenly distributed inside the cells; they can be found not only in the outer mitochondrial membrane, but also in the ER, nuclear envelope and even in cytosol [73-75]. Since Ca^{2+} release from the intracellular stores can affect mitochondrial function, the presence of Bcl-2 in the ER is gradually attracting more interest.

Calcium overload (Fig.5.1) in the mitochondria induces opening of the permeability transition pore (PTP), which results in the release of pro-apoptotic factors [76]. The work carried out by our group shows that calcium responses are indispensable for apoptosis induction via the intrinsic pathway in pancreatic acinar cells [8]. Therefore, Bcl-2 proteins seem to be involved in the regulation of cell death signals in different cell compartments. The role of Bcl-2 proteins in controlling Ca^{2+} fluxes across the plasma membrane is still elusive. It is reported that Bcl-2 could be involved in the modulation of store operated calcium entry [77]. However, the function of Bcl-2 in the regulation of calcium efflux from the cytosol across the plasma membrane has not been elucidated.

The study originated from the initial observation that the loss of Bcl-2 protein resulted in an increased cytosolic Ca^{2+} clearance in pancreatic acinar cells treated with a mixture of 10 μM Tg and 10 μM ACh. Subsequently, this more comprehensive study was undertaken in order to investigate this phenomenon in more detail. The results demonstrate that the Bcl-2 protein is tightly linked with cellular calcium homeostasis. Pancreatic acinar cells without the Bcl-2 protein extrude cytosolic calcium across the plasma membrane more efficiently than wild type cells. In pancreatic acinar cells calcium extrusion across the plasma membrane is mainly dependent on the PMCA.

As the experimental data show, the substitution of $NMDG^{+}$ for Na^{+} in the extracellular solution did not affect Ca^{2+} extrusion across plasma membrane. Since the Na^{+}-Ca^{2+} exchange is negligible in pancreatic acinar cells, it is clear that the effects of Bcl-2 loss are associated with the PMCA. Bcl-2 appears to suppress PMCA-mediated cellular Ca^{2+} extrusion; therefore in Bcl-2 KO cells Ca^{2+} extrusion from the cytosol is more efficient. Furthermore, calcium extrusion is one of the main mechanisms maintaining cytoplasmic calcium levels. Therefore Bcl-2 KO cells have significantly lower resting cytosolic calcium levels than wild type cells. Studies on the AR42J cell line revealed that Bcl-2 overexpression led to reduced resting levels of calcium in the ER and silencing Bcl-2 expression resulted in increased resting $[Ca^{2+}]_{ER}$ [63]. This work provides a new and unexpected role of Bcl-2-dependent control of the PMCA,

although it does not elucidate it completely.

Localization studies indicate that Bcl-2 is widely expressed in both pancreatic acinar cells and AR42J cells [63]. While a substantial proportion of Bcl-2 is localized to the mitochondria, a small amount is also found in the ER in close proximity to the plasma membrane. It is clear that the ER can come very close to the plasma membrane in pancreatic acinar cells; therefore it is not surprising that Bcl-2 can be localized very close to the PMCA. This clearly does not confirm a direct interaction between these two proteins but certainly does not exclude such an interaction.

Finally, the pathophysiological consequences of PMCA regulation by Bcl-2 protein have been investigated. A high concentration of extracellular calcium (5 mM) alone or treated with menadione leads to a substantial increase in necrosis in wild type cells, but not in Bcl-2 KO cells. The survival rate of cells without Bcl-2 is not affected by increased extracellular calcium, but they are more likely to die of apoptosis when treated with menadione alone. Interestingly, necrosis remains at low levels even upon combined treatment with menadione and high calcium. Further study showed that WT cells treated with menadione combined with Caloxin 3A1, compared with WT cells treated with menadione alone, dramatically increased the proportion of necrotic cells. These results provide novel insights into understanding of the functions of Bcl-2 protein. Our data show that Bcl-2 KO cells are protected against the adverse effects of high extracellular Ca^{2+}, because they can extrude cytosolic Ca^{2+} more efficiently than WT cells. Furthermore, loss of Bcl-2 protein strongly promotes apoptosis and at the same time protects against excessive necrosis when cells are challenged with an agent (menadione) generating reactive oxygen species.

Alcohol abuse is the one of main reason of acute pancreatitis. However, the mechanism of how alcohol affects calcium homeostasis and prematurely activates intracellular trypsinogen in pancreatic acinar cells is still unclear. Many Ca^{2+}-permeable channels exert negative feedback on their activity by Ca^{2+} dependent mechanisms. This regulation can either be direct, as the channels themselves have cytosolic Ca^{2+} binding sites, or indirect, mediated through the effects of a Ca^{2+} binding protein such as CaM.

It has been demonstrated that CALP1 and CALP3 mimic the effect of Ca^{2+} through their specific interaction with EF hands of CaM [71]. After they bind to CaM, the CALP/CaM complex can activate phosphodiesterase in the absence of Ca^{2+}. Specifically, these peptides exert their action as Ca^{2+} channel blockers on the Ca^{2+} sensing mechanisms. It has been demonstrated that ethanol in concentrations of10mM can release substantial Ca^{2+} from intracellular stores in two-photon permeabilized acinar cells [72]. This is crucial for pathological intracellular trypsinogen activation in the granule area, which strongly correlates with acute pancreatitis. It is now clear that high concentrations of calmodulin in intact pancreatic acinar cells inhibit ethanol-induced, RyR- and IP_3R-mediated and amplified, Ca^{2+} release from the

intracellular stores, the ER and the acidic stores in the apical area. This explains why ethanol in high concentrations has minor effects on Ca^{2+} homeostasis, but can trigger massive Ca^{2+} release in permeabilized acinar cells without calmodulin.

It has also been shown that re-admission of CaM could abolish the toxic effect of high doses of ethanol (100 mM) and partially inhibit Ca^{2+} release from internal stores in permeabilized cells [72]. The different responses to ethanol between intact and permeabilized cells can be explained by the presence of CaM inside the cells. The evidence supports the view that nonoxidative metabolites of ethanol, fatty acid ethyl esters, are the main substances which have damaging effects on the pancreatic acinar cells [78]. However, it is still unclear how calmodulin inhibits the toxic effects of ethanol.

It was suggested that CaM could inhibit IP_3R opening in order to control Ca^{2+} release [79, 80]. It was also demonstrated that type 2 and 3 IP_3Rs played the crucial role in alcohol-related acute pancreatitis and the Ca^{2+} released from the acid stores was especially important for the trypsinogen activation [72]. Although the mechanism of CaM action is unknown, the protective effect of CaM on the granular store is currently attracting more interest. It has been reported that calmodulin is recruited from the basolateral cytoplasm to the apical region due to the Ca^{2+} spikes in the granular region elicited by hormonal stimulation [81]. Interestingly, excessive doses of hormone stimulation resulting in sustained calcium elevations only lead to a single transient phase of CaM recruitment to the granular pole. In contrast, physiological Ca^{2+} oscillations recruit CaM from the basolateral pole to the granular pole during each repetitive Ca^{2+} spike. This suggests that repetitive Ca^{2+} spiking is important for the CaM recruitment to the apical part of the cells.

This study provides important observations from a medical point of view. Pre-activation of calmodulin by a calcium-like peptide inhibits Ca^{2+} release from the intracellular stores and trypsinogen activation. This could avoid pancreatic acinar cells dying from necrosis which is very dangerous to the whole pancreas tissue. In the meantime, it could switch necrosis to apoptosis which is the safe way of cell death. How the activated calmodulin regulates the IP_3Rs and promotes the apoptosis of pancreatic acinar cells need more investigation. These data indicate that the development and improvement of calmodulin activators could have some therapeutic benefits to pancreatitis.

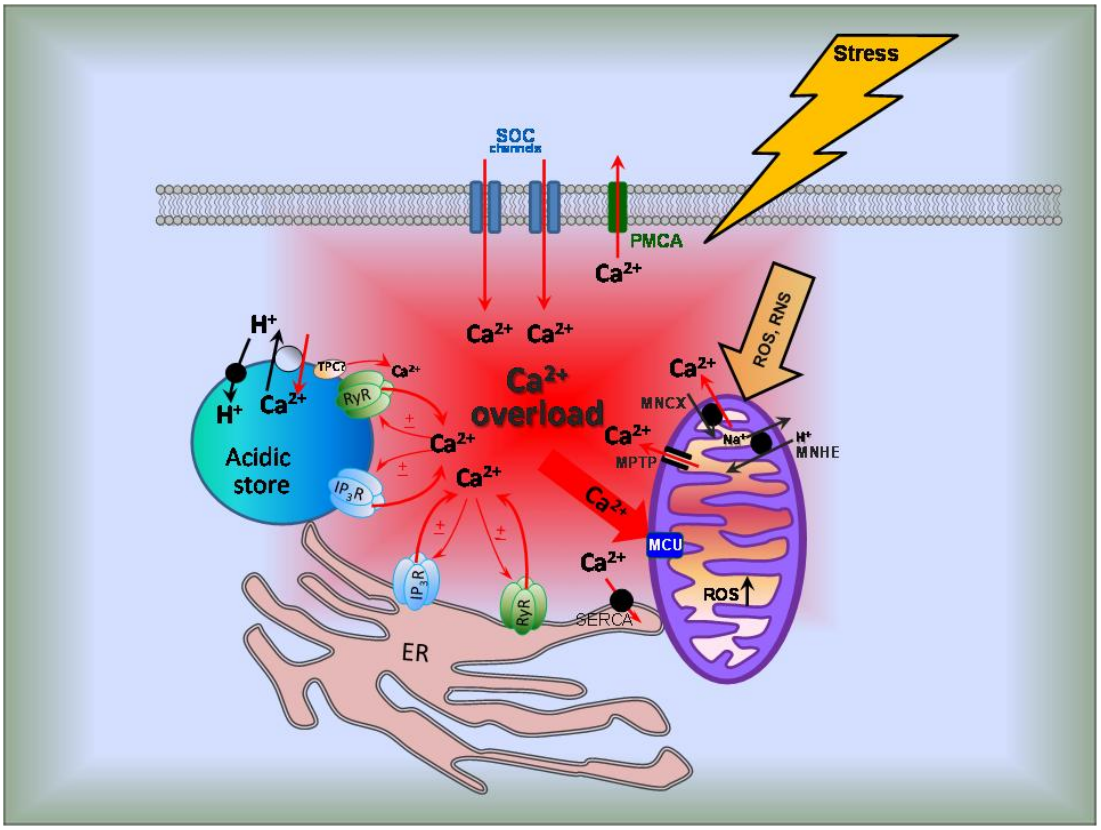


Fig.5.1. Diagram of pathological activity of calcium overload in pancreatic acinar cells.

Hyperstimulation which causes acute pancreatitis can induce pathological stress. This often induces Ca^{2+} release from internal stores. Subsequently, Ca^{2+} entry is followed by Ca^{2+} release. Increased Ca^{2+} entry and decreased Ca^{2+} extrusion can lead sustained cytoplasmic Ca^{2+} response, which promote Ca^{2+} overload and irreversible inhibition of mitochondrial energy production. Prolonged depletion of particularly acidic Ca^{2+} stores eventually leads to pancreatic digestive proenzymes activation and subsequent necrosis. [Adapted from Gerasimenko OV] [82]

Chapter 6:

Conclusions

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This dissertation presents the data on the novel role for the anti-apoptotic Bcl-2 protein in the regulation of Ca^{2+} extrusion and the new data regarding the reduction of pathological Ca^{2+} signals and necrosis by CaM activators.

We found that Bcl-2 regulates calcium extrusion in pancreatic acinar cells via PMCA. The close localization between Bcl-2 and PMCA potentially enables them to interact with each other's functions. Bcl-2 KO cells were much less affected by high extracellular Ca^{2+} and much better protected against necrosis when challenged with the oxidant menadione. The loss of Bcl-2 protein in pancreatic acinar cells promotes apoptosis and protects against necrosis. These results have important patho-physiological implications for developing therapeutic drugs inhibiting Bcl-2 family proteins.

The cell permeable CaM activator peptides used in this study inhibit the activity of certain Ca^{2+} channels. In this study, CALPs were used for pre-activation of calmodulin in intact pancreatic acinar cells. Our results demonstrate that CALPs reduce pathological Ca^{2+} signals, whilst substantially reducing necrosis and potentiating apoptosis in cells treated with the oxidant menadione. CaM activators could potentially be used as drugs to treat acute pancreatitis.

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