Inhibitors of Bcl-2 protein family deplete ER Ca\(^{2+}\) stores in pancreatic acinar cells

Julia Gerasimenko · Pawel Ferdek · Lars Fischer · Anna S. Gukovskaya · Stephen J. Pandol

Received: 12 January 2010 /Revised: 11 June 2010 /Accepted: 17 June 2010 /Published online: 9 July 2010 © The Author(s) 2010. This article is published with open access at Springerlink.com

Abstract Physiological stimulation of pancreatic acinar cells by cholecystokinin and acetylcholine activate a spatial-temporal pattern of cytosolic [Ca\(^{2+}\)] changes that are regulated by a coordinated response of inositol 1,4,5-trisphosphate receptors (IP\(_3\)Rs), ryanodine receptors (RyRs) and calcium-induced calcium release (CICR). For the present study, we designed experiments to determine the potential role of Bcl-2 proteins in these patterns of cytosolic [Ca\(^{2+}\)] responses. We used small molecule inhibitors that disrupt the interactions between prosurvival Bcl-2 proteins (i.e. Bcl-2 and Bcl-x\(_l\)) and proapoptotic Bcl-2 proteins (i.e. Bax) and fluorescence microfluorimetry techniques to measure both cytosolic [Ca\(^{2+}\)] and endoplasmic reticulum [Ca\(^{2+}\)]. We found that the inhibitors of Bcl-2 protein interactions caused a slow and complete release of intracellular agonist-sensitive stores of calcium. The release was attenuated by inhibitors of IP\(_3\)Rs and RyRs and substantially reduced by strong [Ca\(^{2+}\)] buffering. Inhibition of IP\(_3\)Rs and RyRs also dramatically reduced activation of apoptosis by BH3I-2'. CICR induced by different doses of BH3I-2' in Bcl-2 overexpressing cells was markedly decreased compared with control. The results suggest that Bcl-2 proteins regulate calcium release from the intracellular stores and suggest that the spatial-temporal patterns of agonist-stimulated cytosolic [Ca\(^{2+}\)] changes are regulated by differential cellular distribution of interacting pairs of prosurvival and proapoptotic Bcl-2 proteins.

Keywords Pancreas · Pancreatic acinar cell · Acetylcholine · Transport · Signal transduction · Cell death

Introduction

Calcium is the predominant intracellular second messenger in the pancreatic acinar cell mediating its normal physiologic function of digestive enzyme secretion [1]. Calcium also plays a key role in the acinar cell pathobiologies of pancreatitis [2–4]. There are differences in patterns of calcium release from intracellular stores which are responsible for the changes in cytosolic [Ca\(^{2+}\)] observed between physiological and pathobiological conditions in the pancreatic acinar cell. For example, normal physiological stimulation of acinar cells by cholecystokinin (CCK) and acetylcholine causes cytosolic [Ca\(^{2+}\)] transients (oscillations) originating in the apical pole of the cell (the location of the secretory granules) with propagation of each cytosolic [Ca\(^{2+}\)] transient toward the basolateral membrane (wave) without reaching the basolateral membrane. That is, with physiological stimulation the oscillations of cytosolic
[Ca\(^{2+}\)] are contained in the apical pole of the cell [5–7]. In contrast, excessive stimulation of acinar cells with CCK or acetylcholine leads to global and sustained increases in cytosolic [Ca\(^{2+}\)] resulting in cellular pathologies of pancreatitis [2, 3].

The patterns of cytosolic [Ca\(^{2+}\)] changes described above are due to both a differential distribution of calcium release channels on the intracellular calcium stores and the phenomenon of calcium-induced calcium release (CICR). Inositol 1,4,5-trisphosphate receptors (IP\(_3\)R) calcium release channels are concentrated in the apical region [5, 8, 9]. Whereas ryanodine receptors (RyR) are distributed evenly throughout the cell [10–13]. CICR occurs by a mechanism whereby a rise in cytosolic [Ca\(^{2+}\)] facilitates the release of further calcium from intracellular stores [7, 13]. Thus, a small increase in cytosolic [Ca\(^{2+}\)] triggers a greater increase resulting in augmentation of the signal. Several models that have been proposed to explain cytosolic [Ca\(^{2+}\)] spatiotemporal include CICR as a key component [5, 14, 15].

Previously [7], we directly measured CICR sensitivity in the different regions of the acinar cell by locally releasing caged calcium and monitoring for CIRC-induced cytosolic [Ca\(^{2+}\)] waves. Releasing caged calcium in the apical region resulted in a cytosolic [Ca\(^{2+}\)] wave that propagated toward the basal region of the cell. In contrast, CICR could not be initiated by uncaging calcium in the basolateral region of the cell despite the fact that IP\(_3\)Rs and RyRs are present there. IP\(_3\)Rs and RyRs were both necessary for CICR because application of inhibitors specific for each of the receptors prevented CICR stimulated by release of caged calcium. CICR has similarly been observed by others in pancreatic acinar cells [16]. The underlying mechanism of the differential CICR responses in different regions of the cell and the mechanisms regulating CICR are unknown.

Proteins of the Bcl-2 family are known as major regulators of mitochondrial function and mitochondrial-mediated cell death pathways [17, 18]. The Bcl-2 family proteins also participate in calcium signalling [19]. Based on their function and structure, the Bcl-2 family proteins are divided into three groups [18, 20, 21], namely, prosurvival proteins such as Bcl-2 and Bcl-xL containing four BH domains (BH1–BH4); proapoptotic proteins such as Bax, Bak and tBid containing three homologous BH domains (BH1–BH3); and pro-apoptotic proteins such as Bad, Bim and Puma containing one BH3 domain only. In relation to mitochondrial function, the prosurvival Bax and Bak form channels in the outer membrane of the mitochondria resulting in release of proapoptogenic signals such as cytochrome c. BH3-only proteins Bad, Bim and Puma promote the formation of Bax/Bak channels. Proteins Bcl-xL and Bcl-2 bind to and sequester the proapoptotic proteins resulting in the inhibition of apoptosis.

There is substantial evidence that members of the Bcl-2 family of proteins regulate calcium content and release from intracellular stores [19]. However, their roles in CICR have not been determined. During the past several years, small-molecule inhibitors of prosurvival Bcl-2/Bcl-xL have been developed and shown to cause dissociation of prosurvival and proapoptotic Bcl-2 and initiate apoptosis in cancer cells [22, 23].

Because of the previous reports showing potential roles for Bcl-2 proteins in regulating calcium stores and the possibility that Bcl-2 protein interactions could explain the differential sensitivity of stores to CICR, we undertook a set of studies using two small molecular inhibitors that cause dissociation of prosurvival and proapoptotic Bcl-2 proteins to determine the role of such Bcl-2 protein interactions in the mechanism of acinar cell calcium metabolism.

**Materials and methods**

Antibodies against Bcl-xL, Bcl-2 and p44/42 MAP kinase (ERK1/2) were from Cell Signalling (Beverly, MA); Bax and protein disulfide isomerase (PDI) from Santa Cruz Biotechnology (Santa Cruz, CA); COX IV, from Molecular Probes (Eugene, OR). CCK-8, from American Peptide (Sunnyvale, CA). The Bcl-xL/Bcl-2 inhibitor 3-iodo-5-chloro-N-[2-chloro-5-(4chlorophenyl)-sulphonyl]phenyl]-2-hydroxybenzamide (BH3I-2′) was from Calbiochem (La Jolla, CA); ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA 14-1), from ALEXIS Biochemicals (San Diego, CA). Fluo-4 and Fluo-5AM esters were from Molecular Probes/Invitrogen (Eugene, OR). Other reagents were from Sigma Chemical (St. Louis, MO).

**Isolation of pancreatic acinar cells** Freshly isolated mouse pancreatic acinar cells, obtained from male CD-1 mice, were prepared using collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA) digestion as previously described [24]. Pancreata were obtained from adult male mice (CD1) that had been killed by cervical dislocation in accordance with the Animals (Scientific Procedures) Act of 1986 (UK). Training and oversight of procedures were conducted by competent personnel from the University of Liverpool (in compliance with national requirements). The standard extracellular solution used throughout cell isolation and during all experiments contained (in mM): NaCl 140, KCl 4.7, CaCl\(_2\) 1, MgCl\(_2\) 1.13, glucose 10, HEPES 10 (adjusted to pH 7.2 with NaOH). In some experiments, where indicated, CaCl\(_2\) was omitted from the extracellular solution. All experiments were performed at room temperature and cells were used within 3–4 h after isolation.
Subcellular fractionation Subcellular fractionation of pancreatic tissue was performed by differential centrifugation as described in [25, 26]. The dissected pancreas was homogenised in 8 ml of homogenization buffer with five full strokes, and the nuclei and cell debris were sedimented at 150×g. The post-nuclear supernatant was centrifuged at 1,300×g, and the pellet containing zymogen granules was discarded. The supernatant was further centrifuged at 12,000×g, and both the 12,000×g pellet and supernatant were collected. Total protein in the fractions was measured by Bradford assay (Bio-Rad Laboratories, Hercules, CA).

Immunoprecipitation Tissue was lysed in a buffer containing 10 mM HEPES, pH 7.4, 140 mM KCl, 5 mM MgCl2, 0.5 mM EGTA, 2% CHAPS containing 1 mM dithiothreitol, 10 µg/ml each leupeptin and aprotinin, 1 mM PMSF [27]. The lysates were clarified by centrifugation, and 500 µg of protein was subjected to overnight immunoprecipitation with either Bcl-xL or Bcl-2 antibody at 4°C using Catch and Release Reversible Immunoprecipitation System from Millipore (Billerica, MA).

Western blot analysis Western blot analysis was performed on cell homogenates, subcellular fractions and immunoprecipitates as previously described [24, 28]. Proteins were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. Nonspecific binding was blocked by 1-h incubation of the membranes in 5% (w/v) nonfat dry milk in Tris-buffered saline (pH 7.5). Blots were then incubated for 2 h at room temperature (or overnight at 4°C) with primary antibodies in the antibody buffer containing 1% (w/v) nonfat dry milk in TTBS (0.05% (v/v) Tween-20 in Tris-buffered saline), washed three times with TTBS, and finally incubated for 1 h with a peroxidase-labeled secondary antibody in the antibody buffer. Blots were developed for visualisation using enhanced chemiluminescence detection kit (Pierce, Rockford, IL).

Cytosolic Ca2+ measurements For fluorescent imaging of Ca2+, cells were loaded with 3 µM Fluo-4 AM. Loading was carried out at room temperature for 30 min in darkness. Once loaded, cells were placed onto glass coverslips and continuously perfused with extracellular solution from a gravity-fed perfusion system. Confocal imaging was carried out using a Leica SP2 MP system (Leica Microsystems AG, Wetzlar, Germany) with a ×63 1.2 NA objective. Fluorescence was excited at 476 nm and emission was collected between 500 and 550 nm. An analysis of images was performed using Leica software.

Two-photon permeabilization and measurements of Ca2+ in intracellular ER [Ca2+]store measurements were performed as described previously [29]. Briefly, cells were loaded with 5–7.5 µM Fluo-5N AM, for 45 min at 36.5°C, and then transferred onto poly-L-lysine-coated coverslips in a perfusion chamber. Cells were washed with an intracellular K-Hepes solution, containing (mM): KCl, 127; NaCl, 20; Hepes KOH, 10; ATP, 2; MgCl2, 1; EGTA, 0.1; CaCl2 0.05; pH 7.2; 291 mosmol/l. Thereafter, cells were permeabilized using a two-photon microscope, as previously described [29]. In the [Ca2+] clamp experiments 10 mM BAPTA and 2 mM CaCl2 were included into K-Hepes solution. Cells were observed using a Leica SP2 MP dual two-photon microscope using excitation 476 nm and emission at 500–600 nm.

Overexpression of Bcl-2 Protein in AR42J Cells Rat pancreatic tumour cell line AR42J was maintained in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 50 µg/ml gentamycin and 2.5 µg/ml fungizone at 37°C 5% CO2.

Cells were transfected with pEGFP-C1 plasmid containing human Bcl-2 insert obtained through Addgene (plasmid 17999) using PromoFectin reagent (PromoKine) according to the manufacturer's protocol. After 48–72 h cells were loaded with 5 µM fura-2 AM (Invitrogen) at 37°C for 1 h in NaHepes 1 mM Ca2+. Fura-2 ratiometric measurements of intracellular calcium changes were performed by sequential excitation with 340 and 380 nm.

Apoptosis Measurements with caspase substrate Measurements using generic fluorescent caspase substrate rhodamine 110 bis-L-aspartic acid amide (Invitrogen) were conducted as described previously [43]. Briefly, isolated pancreatic acinar cells were washed and suspended in calcium-free buffer solution (140 mM NaCl, 1.13 mM MgCl2, 4.7 mM KCl, 10 mM glucose, 2 mM EDTA, 10 mM HEPES, pH 7.2). Cells were then loaded with caspase substrate (10 µM) at room temperature for 20 min. After loading, cells were washed and treated with BH3I-2’ (15 µM) in the absence or in the presence of ruthenium red (10 µM) and 2-APB (100 µM). Cells were analysed using confocal microscopy (excitation 488 nm, emission 505–543 nm).

Results

In order to determine the location of Bcl-2 and Bcl-xl in pancreatic acinar cells and the effects of Bcl-2/Bcl-xl inhibitors on associations of Bcl-2 family proteins, we performed the series of Western blot analyses illustrated in Fig. 1. For the experiment shown in Fig. 1a, we determined differential localization of Bcl-2 and Bcl-xl in a two post-nuclear fractions of pancreatic tissue-the 12,000×g pellet
and 12,000×g supernatant. We monitored organelle markers COX IV that is specific for mitochondria and PDI that is specific for endoplasmic reticulum. The results (Fig. 1a) show that the 12,000×g pellet fraction contains mitochondria and endoplasmic reticulum as well as both Bcl-2 and Bcl-xl; and that the 12,000×g supernatant fraction contains no mitochondria but does contain endoplasmic reticulum as well as Bcl-2 and Bcl-xl. Importantly, the supernatant fraction with endoplasmic reticulum devoid of mitochondria had a greater concentration of the Bcl-2 proteins compared to the mitochondrial containing fraction indicating a potential role for Bcl-2 proteins in endoplasmic reticulum function.

The immunoprecipitation/Western blot in Fig. 1b, c were performed to determine the effects of the two putative Bcl-2/Bcl-xl inhibitors, 5 μM BH3I-2' and 30 μM HA14-1, on associations of a BH3-only Bcl-2 family member (Bax) and Bcl-2 or Bcl-xl. Disruptions of the interactions are a measure of inhibitory activity. As shown in these two figures (Fig. 1b, c) both BH3I-2' and HA14-1 disrupt the interactions of Bax with Bcl-2 and Bcl-xl confirming their inhibitory activity in pancreatic tissue.

To investigate the role of Bcl-2/Bcl-xl proteins on calcium release from the internal stores we loaded freshly isolated pancreatic acinar cells with the AM ester form of calcium sensitive fluorescent cytosolic dye Fluo-4. All experiments shown in Fig. 2 were performed in the nominally calcium-free solution in order to focus on release of calcium from internal stores only. Fluorescence was monitored using confocal microscopy. Inhibition of Bcl-2/ Bcl-xl was induced by either 5 μM BH3I-2' or 30 μM HA14-1. Each of the agents caused a slow transient increase of [Ca^{2+}]) in the cytosol followed by partial return of cytosolic [Ca^{2+}] toward baseline levels and plateau formation (Fig. 2a, b). Subsequent addition of a supramaximal dose of CCK was unable to further release calcium (Fig. 2a, b) suggesting that the content of internal calcium stores in each case was substantially reduced by treatments with BH3I-2' and HA14-1.

In contrast to the observations with BH3I-2' and HA14-1, application of 5 nM CCK in the absence of external calcium produced a rapid and transient [Ca^{2+}] rise in the cytosol with complete recovery of [Ca^{2+}] to the basal level; and the subsequent application of the Bcl-2/Bcl-xl inhibitors induced very slow dose dependent increases in cytosolic [Ca^{2+}] followed by a plateau in cytosolic [Ca^{2+}] (Fig. 2c, d). Formation of the plateau in cytosolic [Ca^{2+}] induced by Bcl-2 inhibitors shows that there is a new equilibrium in cellular calcium level probably resulting from the balance of Ca^{2+} influx and Ca^{2+} extrusion.

Experiments shown in Figs. 3 and 4 were performed to measure calcium changes in intracellular stores using two-photon fluorescence microscopy and permeabilized pancreatic acinar cells loaded with calcium sensitive low affinity indicator Fluo-5N AM as we described previously [29]. Application of 5 μM BH3I-2' caused a reduction in fluorescence (18.5%±2.2 SE) indicating a decrease of the calcium content in internal stores (Figs. 3a and 4g; n=12). Similarly, 30 μM HA14-1 decreased Ca^{2+} in the intracellular stores (Figs. 3b and 4g; 21%±1.5 SE, n=10). Pretreatment of the permeabilized cells with mixture of 10 μM rotenone and 10 μM oligomycin did not prevent BH3I-2'- and HA14-1-dependent calcium loss indicating that the effects of BH3I-2' and HA14-1 were independent of mitochondrial or ATP effects that these inhibitors might have in the cells (Figs. 3c, d and 4g).

When [Ca^{2+}] was clamped in the buffer with 10 mM BAPTA/2 mM CaCl_2 in order to block calcium-induced calcium release (CICR) from the stores, the effects of both BH3I-2' or HA14-1 were markedly inhibited (Figs. 3e, f and 4g; 6.8%±0.3 SE, n=6 for BH3I-2'; 6.1%±0.3 SE, n= 6 for HA14-1).
A blocker of IP$_3$Rs, 2-APB (100 μM), reduced the amplitude of BH3I-2' and HA14-1-induced Ca$^{2+}$ release from internal stores but did not inhibit the responses completely (Fig. 4a, b, g; 13%±1.9 SE, n=8 for BH3I-2'; 11.7%±0.9 SE, n=5 for HA14-1). Also, inhibition of RyRs with ruthenium red (10 μM) partially inhibited BH3I-2'- and HA14-1-induced reduction of Ca$^{2+}$ in the stores (Fig. 4c, d, g; 12.5%±1.2 SE, n=5 for BH3I-2'; 12%±1.4 SE, n=5 for HA14-1). A mixture of 2-APB (100 μM) and ruthenium red (10 μM) inhibited Bcl-2/Bcl-xL inhibitor-induced calcium release to a greater extent than either agent alone although the inhibition was not complete (Fig. 4e–g; 8.8%±0.5 SE, n=5 for BH3I-2'; 7.3%±0.9 SE, n=5 for HA14-1).

The results shown in Figs. 2, 3 and 4 suggest that inhibition of Bcl-2 proteins by both BH3I-2' and HA14-1 induces Ca$^{2+}$ release from the ER stores. IP$_3$Rs and RyRs are both partially involved in the Ca$^{2+}$ release but their role seems to be limited to amplification of the leak probably by CICR.

To determine if CICR is dependent on antiapoptotic proteins we overexpressed Bcl-2 in AR42J cells. Application of BH3I-2' in the range between 2 and 15 μM induced calcium release measured with Fura-2 and clear a CICR component in control cells. However, in Bcl-2 overexpressing cells the increasing phases of responses were substantially diminished (Fig. 5 a, b). Responses to 1 μM of BH3I-2' were markedly decreased in Bcl-2 overexpressing cells (P>0.39, n=16) so that the response was essentially abolished. These further support our suggestion that Bcl-2 protein interactions are an essential component the CICR.

Because Bcl-2 family proteins play a major role in apoptosis, we measured the apoptosis induction by Bcl-2 family inhibitor BH3I-2' in three series of independent experiments with 20–80 cells each. Fifteen micromolars of BH3I-2' induced apoptosis in the majority of treated cells (58.4±2.5%). In the presence of the mixture of inhibitors of IP$_3$Rs (2-APB (100 μM) and RyRs (ruthenium red (10 μM)) percentage of apoptotic cells was reduced to 15.8±0.7%, only slightly greater than control values (7.3±3.7%).
These data demonstrate the importance of Bcl-2-dependent CICR-type calcium release from intracellular stores in the mechanism of apoptosis.

Discussion

The results of the current study demonstrate that the endoplasmic reticulum of the pancreatic acinar cell contains significant quantities of Bcl-2 family proteins and that the two small molecule inhibitors of Bcl-2/Bcl-xl with markedly dissimilar molecular structures cause dissociation of proapoptotic Bax from prosurvival Bcl-2 and Bcl-xl. Importantly, this dissociation of Bcl-2 proteins was associated with the ability of these agents to cause release of calcium from intracellular stores of the pancreatic acinar cell. Furthermore, the treatment with the Bcl-2/Bcl-xl inhibitors had identical and specific effects on calcium releasing receptors strongly supporting a role for Bcl-2 proteins in regulating these receptors.

We previously showed [30] that Bcl-2/Bcl-xl inhibitors cause depolarization of pancreatic mitochondria and stimulate cytochrome c release. However, the effects described in this report were not due to the potential effects on mitochondrial energetics because complete inhibition of mitochondrial function with a combination of rotenone and oligomycin did not prevent reduction of calcium content induced by both inhibitors.

Importantly, the investigation of the mechanism of the effect of the Bcl-2/Bcl-xl inhibitors showed that both IP3R and RyR functions are necessary for their effects on release of calcium from intracellular stores. That is, the IP3R and RyR blockers, 2-APB and ruthenium red [31], respectively, each partially prevented the decrease of calcium in stores caused by the inhibitors. Further, the effects of the blockers of IP3Rs and RyRs were additive suggesting a role for both types of calcium releasing receptors in the mechanism of effect of the Bcl-2/Bcl-xl inhibitors. Interestingly, the effect of inhibitors in intact cells usually causes substantial calcium release with a long calcium plateau even in the calcium-free medium. Pancreatic acinar cells are known to respond to stimulation in calcium-free solution for very long time [32]. A calcium plateau in similar conditions has
been observed previously [33], particularly in relation to inhibited calcium extrusion [34]. By adding a relatively small amount of calcium chelator EGTA (100 μM) we completely removed this effect. Therefore, calcium release from intracellular stores is the major effect of application of Bcl-2 family inhibitors while the calcium plateau is a secondary minor event.

The effect of the Bcl-2/Bcl-xl inhibitors on calcium release from intracellular stores was also nearly completely prevented by clamping the [Ca^{2+}] surrounding the store compartment. The best interpretation of the combined findings listed here is that dissociating proapoptotic Bcl-2 proteins such as Bax from prosurvival Bcl-2 proteins Bcl-2 and Bcl-xl increases the sensitivity of IP3Rs and RyRs to activation by calcium, a mechanism of CIRC in pancreatic acinar cells described previously [7, 16]. Of note, this sensitization to calcium did not require addition of ligands for these receptors showing that the alteration of their
acinar cells in nominally calcium-free solution in the presence of 100 μM EGTA. Cells were loaded with 3 μM Fluo-4 AM (n=7).

d Measurements of general caspase activation induced by 15 μM BH3I-2' in the presence and in the absence of the mixture of 2-APB (100 μM) and ruthenium red (10 μM). Cells were loaded with Rhodamine 110 in the calcium-free buffer in the presence of 2 mM EGTA. Data represent percentage of apoptotic cells in control (7.3±0.8%) or without the mixture of 2-APB and ruthenium red (58.4±2.5%)

calcium sensitivity alone can activate their release function. This finding is reminiscent of CICR in the apical pole of the acinar cell where release of caged calcium alone can activated CICR [7]. Also, the large effects of the inhibitors suggest a key role for the association of antiapoptotic and proapoptotic in regulating calcium signalling.

Over expressing Bcl-2 in AR42J cells substantially reduced the [Ca^{2+}] response to BH3I-2' confirming our conclusions about the role of Bcl-2 in the response. Importantly, these responses highly influence cell fate, i.e. by inhibiting IP3Rs and RyRs, apoptosis was dramatically reduced. Interestingly, the inhibition of IP3Rs and RyRs neither completely blocked calcium release induced by Bcl-2 family inhibitors nor completely blocked apoptosis induction.

Although there are no previous studies we are aware of, that would demonstrate any role of Bcl-2 proteins in CICR, there are numerous studies showing a role of Bcl-2 family members in calcium metabolism [35–42] and for review [19]. Previous findings provide certain insights indirectly linked to the CICR in the present study, such as the demonstration that Bcl-2 and/or Bcl-xl physically bind to the IP3R and alter its ability to release calcium [41, 42]. In one study [42], Bcl-xl was found to bind directly to the C-terminal domain of IP3R increasing its sensitivity to IP3. This effect was prevented by addition of Bax or tBid. Taken together with our results showing that dissociation of Bcl-xl from Bax is associated with calcium release from intracellular stores, increasing evidence suggests a model whereby prosurvival Bcl-2 and Bcl-xl regulate calcium releasing channels as a function of their association with one or more proapoptotic Bcl-2 proteins. When associated with proapoptotic proteins, the prosurvival Bcl-2 proteins inhibit the calcium releasing channels. On the other hand when dissociated from proapoptotic Bcl-2 proteins, Bcl-2 and Bcl-xl increase the sensitivity of the channels to calcium release.

In conclusion, the present findings show that a treatment that dissociates proapoptotic Bcl-2 sequestered by prosurvival Bcl-2 proteins increases the sensitivity of IP3Rs and RyRs for activation by calcium. Considering previous studies showing that calcium activation of these receptors

---

Fig. 5 CICR after application of BH3I-2' is substantially reduced in Bcl-2 overexpressing cells. a Representative traces of responses to 15 μM BH3I-2' in control (black line) and in Bcl-2 overexpressing AR42J cells (grey line). b Rates of the increasing phase of responses to BH3I-2' in control (white bars) and Bcl-2 overexpressing AR42J cells (grey bars). Significant difference marked by asterisks and number sign for 2 μM (P<0.036, n=19), 5 μM (P<0.032, n=17) and 15 μM (P<0.041, n=19) of BH3I-2' as compared to control (n>19 for each concentration). c Typical cytosolic [Ca^{2+}] response induced by 5 μM BH3I-2' in freshly isolated pancreatic acinar cells in nominally calcium-free solution in the presence of 100 μM EGTA. Cells were loaded with 3 μM Fluo-4 AM (n=7).
(CICR) is normally restricted to the apical pole of the acinar cell and that CICR is hypothesised to underlie physiological cytosolic [Ca^{2+}] oscillations and waves, it is tempting to speculate that there is a differential distribution of associations between Bcl-2 proteins in the apical and basolateral regions of the acinar cell to account for these phenomena. Further, changes in distribution and/or associations between Bcl-2 proteins could account for the global increases of [Ca^{2+}] that occur during pathologic conditions.

Acknowledgements Department of Veterans Affairs, Greater Los Angeles Healthcare System and USC-UCLA Southern California Research Center for ALPD and Cirrhosis (P60 AA11999) and MRC Programme Grant (G8801575) and by the Wellcome Trust Prize Ph.D. studentship to P.F.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

1. Petersen OH, Tepikin AV (2008) Polarized calcium signaling in exocrine gland cells. Annu Rev Physiol 70:273–299
2. Sutton R, Petersen OH, Pandol SJ (2008) Pancreatitis and calcium signalling: report of an international workshop. Pancreas 36:e1–e14
3. Mukherjee R, Criddle DN, Gukovskaya A, Pandol S, Petersen OH, Sutton R (2008) Mitochondrial injury in pancreatitis. Cell Calcium 44:14–23
4. Pandol SJ, Saluja AK, Imrie CW, Banks PA (2007) Acute pancreatitis: bench to the bedside. Gastroenterology 133:1056
5. Kasai H, Li YX, Miyashita Y (1993) Subcellular distribution of Ca^{2+} release channels underlying Ca^{2+} waves and oscillations in exocrine pancreas. Cell 74:669–677
6. Toescu EC, Gallacher DV, Petersen OH (1994) Identical regional mechanisms of intracellular Ca^{2+} concentration increase during polarized agonist-evoked Ca^{2+} response in pancreatic acinar cells. Biochem J 304(1):313–316
7. Ashby MC, Craske M, Park MK, Gerasimenko OV, Burgoyne RD, Petersen OH, Tepikin AV (2002) Localized Ca^{2+} uncaging reveals polarized distribution of Ca^{2+}-sensitive Ca^{2+} release sites: mechanism of unidirectional Ca^{2+} waves. J Cell Biol 158:283–292
8. Thom P, Lawrie AM, Smith PM, Gallacher DV, Petersen OH (1993) Local and global cytosolic Ca^{2+} oscillations in exocrine cells evoked by agonists and inositol trisphosphate. Cell 74:661–668
9. Nathanson MH, Fallon MB, Padfield PJ, Maranto AR (1994) 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 269:4693–4696
10. Leite MF, Dranoff JA, Gao L, Nathanson MH (1999) Expression and subcellular localization of the ryanodine receptor in rat pancreatic acinar cells. Biochem J 337( Pt 2):305–309
11. Straub SV, Giovannucci DR, Yule DJ (2000) Calcium wave propagation in pancreatic acinar cells: functional interaction of inositol 1, 4, 5-trisphosphate receptors, ryanodine receptors, and mitochondria. J Gen Physiol 116:547–560
12. Fitzsimmons TJ, Gukovskiy I, McRoberts JA, Rodriguez E, Lai FA, Pandol SJ (2000) Multiple isoforms of the ryanodine receptor are expressed in rat pancreatic acinar cells. Biochem J 351:265–271
13. Ashby MC, Tepikin AV (2002) Polarized calcium and calmodulin signaling in secretory epithelia. Physiol Rev 82:701–734
14. Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signalling. Nature 341:197–205
15. Wakui M, Ospichuk YY, Petersen OH (1990) Receptor-activated cytoplasmic Ca^{2+} spiking mediated by inositol trisphosphate is due to Ca^{2+}-induced Ca^{2+} release. Cell 63:1025–1032
16. Leite MF, Burgstahler AD, Nathanson MH (2002) Ca^{2+} waves require sequential activation of inositol trisphosphate receptors and ryanodine receptors in pancreatic acini. Gastroenterology 122:415–427
17. Cheng EH, Wei MC, Weiler S, Flavell RA, Mak TW, Lindsten T, Korsmeyer SJ (2001) BCL-2, BCL-XL sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 8:705–711
18. Kim H, Rafiuddin-Shah M, Tu HC, Jeffers JR, Zambetti GP, Hsieh JJ, Cheng EH (2006) Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. Nat Cell Biol 8:1348–1358
19. Rong Y, Distelhorst CW (2008) Bcl-2 protein family members: versatile regulators of calcium signaling in cell survival and apoptosis. Annu Rev Physiol 70:73–91
20. Petros AM, Olejniczak ET, Fesik SW (2004) Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta 1644:83–94
21. Lima D, Sankaramakrishnan R (2008) Anti-apoptotic Bcl-XL protein in complex with BH3 peptides of pro-apoptotic Bak, Bad, and Bim proteins: comparative molecular dynamics simulations. Proteins 73:492–514
22. Kang MH, Reynolds CP (2009) Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. Clin Cancer Res 15:1126–1132
23. Azmi AS, Mohammad RM (2009) Non-peptidic small molecule inhibitors against Bcl-2 for cancer therapy. J Cell Physiol 218:13–21
24. Mareninova OA, Sung KF, Hong P, Lugea A, Pandol SJ, Gukovsky I, Gukovskaya AS (2006) Cell death in pancreatitis: caspases protect from necrotizing pancreatitis. J Biol Chem 281:3370–3381
25. Saluja A, Saito I, Saluja M, Howlihan MJ, Powers RE, Meldolesi J, Steer M (1985) In vivo rat pancreatic acinar cell function during supramaximal stimulation with caerulein. Am J Physiol 249:G702–G710
26. Saluja A, Hashimoto S, Saluja M, Powers RE, Meldolesi J, Steer ML (1987) Subcellular redistribution of lysosomal enzymes during caerulein-induced pancreatitis. Am J Physiol 253:G508–G516
27. Zhai D, Jin C, Huang Z, Satterthwait AC, Reed JC (2008) Differential regulation of Bax and Bak by anti-apoptotic Bcl-2 family proteins Bcl-B and Mcl-1. J Biol Chem 283:9580–9586
28. Sung KF, Odinkova IV, Mareninova OA, Rakonczay Z Jr, Hegyi P, Pandol SJ, Gukovsky I, Prosurvival GAS (2009) Bcl-2 proteins stabilize pancreatic mitochondria and protect against necrosis in experimental pancreatitis. Exp Cell Res 315:1975–1989
29. Gerasimenko JV, Sherwood M, Tepikin AV, Petersen OH, Gerasimenko OV (2006) NAADP, cADPR and IP3 all release Ca^{2+} from the endoplasmic reticulum and an acidic store in the secretory granule area. J Cell Sci 119:226–238
30. Sung KF, Odinkova IV, Mareninova OA, Rakonczay Z Jr, Hegyi P, Pandol SJ, Gukovsky I, Gukovskaya AS (2009) Prosurvival Bcl-2 proteins stabilize pancreatic mitochondria and protect against necrosis in experimental pancreatitis. Exp Cell Res 315:1975–1989
31. Gerasimenko JV, Flowerdew SE, Voronina SG, Sukhomlin TK, Tepikin AV, Petersen OH, Gerasimenko OV (2006) Bile acids...
induce Ca2+ release from both the endoplasmic reticulum and acidic intracellular calcium stores through activation of inositol trisphosphate receptors and ryanodine receptors. J Biol Chem 281:40154–40163

32. Xu X, Zeng W, Diaz J, Lau KS, Gukovskaya AC, Brown RJ, Pandol SJ, Mualllem S (1997) nNOS and Ca2+ influx in rat pancreatic acinar and submandibular salivary gland cells. Cell Calcium 22:217–228

33. Tortorici G, Zhang BX, Xu X, Mualllem S (1994) Compartmentalization of Ca2+ signaling and Ca2+ pools in pancreatic acini. Implications for the quantal behavior of Ca2+ release. J Biol Chem 269:29621–29628

34. Toescu EC, Petersen OH (1995) Region-specific activity of the plasma membrane Ca2+ pump and delayed activation of Ca2+ entry characterize the polarized, agonist-evoked Ca2+ signals in exocrine cells. J Biol Chem 270:8528–8535

35. Foyouzi-Youssefi R, Arnaudeau S, Borner C, Kelley WL, Tschopp J, Lew DP, Demaurex N, Krause KH (2000) Bel-2 decreases the free Ca2+ concentration within the endoplasmic reticulum. Proc Natl Acad Sci U S A 97:5723–5728

36. Li C, Fox CJ, Master SR, Bindokas VP, Chodosh LA, Thompson CB (2002) Bcl-X(L) affects Ca2+ homeostasis by altering expression of inositol 1, 4, 5-trisphosphate receptors. Proc Natl Acad Sci U S A 99:9830–9835

37. Scorrano L, Oakes SA, Opferman JT, Cheng EH, Sorcinelli MD, Pozzan T, Korsmeyer SJ (2003) BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. Science 300:135–139

38. Zong WX, Li C, Hatzivassiliou G, Lindsten T, Yu QC, Yuan J, Thompson CB (2003) Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol 162:59–69

39. Oakes SA, Opferman JT, Pozzan T, Korsmeyer SJ, Scorrano L (2003) Regulation of endoplasmic reticulum Ca2+ dynamics by proapoptotic BCL-2 family members. Biochem Pharmacol 66:1335–1340

40. Palmer AE, Jin C, Reed JC, Tien RY (2004) Bel-2-mediated alterations in endoplasmic reticulum Ca2+ analyzed with an improved genetically encoded fluorescent sensor. Proc Natl Acad Sci U S A 101:17404–17409

41. Chen R, Valencia I, Zhong F, McColl KS, Roderick HL, Bootman MD, Berridge MJ, Conway SJ, Holmes AB, Mignery GA, Velez P, Distelhorst CW (2004) Bel-2 functionally interacts with inositol 1, 4, 5-trisphosphate receptors to regulate calcium release from the ER in response to inositol 1, 4, 5-trisphosphate. J Cell Biol 166:193–203

42. White C, Li C, Yang J, Petrenko NB, Madesh M, Thompson CB, Foskett JK (2005) The endoplasmic reticulum gateway to apoptosis by Bcl-X(L) modulation of the InsP3R. Nat Cell Biol 7:1021–1028

43. Baumgartner HK, Gerasimenko JV, Thorne C, Ferdek P, Pozzan T, Tepikin AV, Petersen OH, Sutton R, Watson AJ, Gerasimenko OV (2009) Calcium elevation in mitochondria is the main Ca2+ requirement for mitochondrial permeability transition pore (mPTP) opening. J Biol Chem 284(31):20796–20803