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SIGNALING AND CELL PHYSIOLOGY

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

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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_3Rs), 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-xl) 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_3Rs and RyRs and substantially reduced by strong [Ca^{2+}] buffering. Inhibition of IP_3Rs 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

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[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_3Rs) calcium release channels are concentrated in the apical region [5, 8, 9]. Whereas ryanodine receptors (RyRs) 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_3Rs and RyRs are present there. IP_3Rs 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 proteins 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 isomerise (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-ehtoxy-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 $MgCl_2$, 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 Ca^{2+} measurements For fluorescent imaging of Ca^{2+}, 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 Ca^{2+} in intracellular ER [Ca^{2+}] 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; $MgCl_2$, 1; EGTA, 0.1; $CaCl_2$ 0.05; pH 7.2; 291 mosmol/l. Thereafter, cells were permeabilized using a two-photon microscope, as previously described [29]. In the [Ca^{2+}] clamp experiments 10 mM BAPTA and 2 mM $CaCl_2$ 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% CO_2.

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 Ca^{2+}. 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

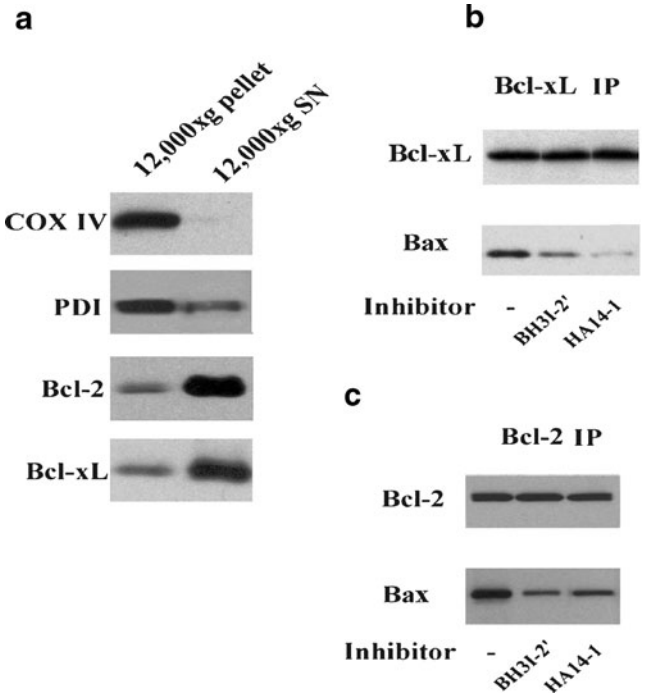

Fig. 1 Bcl-2 and Bcl-xL are present in the ER fraction of acinar cells and release bound Bax with addition of inhibitors 5 μM BH3I-2′ and 30 μM HA14-1. **a** Pancreas was homogenised and postnuclear supernatant was first centrifuged at 1,300×*g*. The pellet enriched in zymogen granules was removed and the supernatant was further centrifuged at 12,000×*g*. Both the pellet and supernatant (*SN*) were analysed using Western blot for the presence of the mitochondrial marker COX IV, ER marker PDI and Bcl-2, and Bcl-xL. **b** and **c** Pancreatic acini were incubated in the presence or absence of 5 μM BH3I-2′ or 30 μM HA14-1 for 1 h followed by cell lysis and immunoprecipation with antibodies to (**b**) Bcl-xL or (**c**) Bcl-2. Immunoprecipitates were probed with antibodies against Bcl-xL, Bcl-2 or Bax. The results are representative of 2 experiments which gave the same results

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 HA14-1disrupt 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).

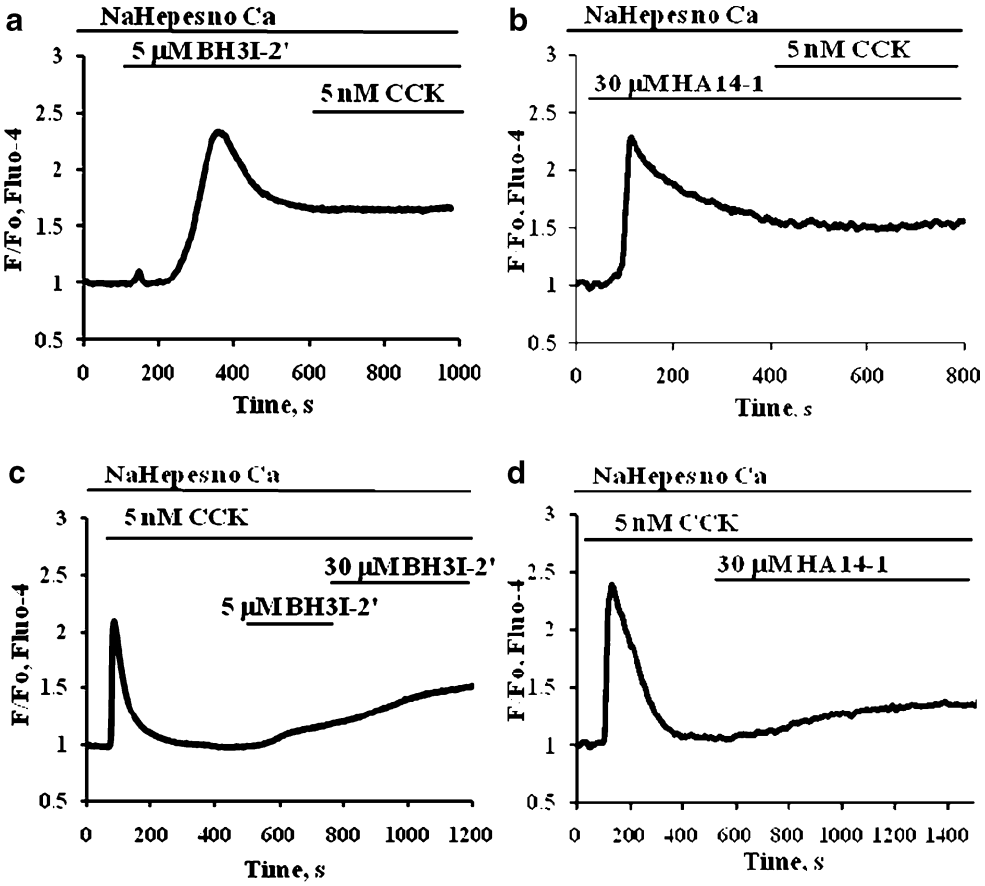

Fig. 2 Cytosolic [Ca^{2+}] responses to application of Bcl-2/Bcl-xL inhibitors –BH3I-2′ and HA14-1 in pancreatic acinar cells. Experiments were performed in Fluo-4 loaded acinar cells incubated in the absence of external $CaCl_2$. **a** Typical calcium response in the cytosol induced by application of 5 μM BH3I-2′ in freshly isolated pancreatic acinar cells. A subsequent application of 5 nM CCK did not produce any additional response. **b** Typical trace of [Ca^{2+}] response in the cytosol elicited by application of 30 μM HA14-1 followed by addition of 5 nM CCK. **c** The 5 nM CCK elicited a global [Ca^{2+}] response in the cytosol. Subsequent additions of 5 μM and 30 μM BH3I-2′ induced slow elevations of Ca^{2+} and plateau formation **d** The 5 nM CCK elicited global [Ca^{2+}] response in the cytosol. Additions of 30 μM HA14-1 induced slow elevations of [Ca^{2+}] and plateau formation

A blocker of IP_3Rs, 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_3Rs 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 with 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.

We have also performed experiments to further confirm that calcium responses we observed with BH3I-2′ were due to release from the internal stores. 5 μM of BH3I-2′ was applied to pancreatic acinar cells in calcium free solution and 100 μM of the calcium chelator EGTA (Fig. 5c, n=7). The responses to 5 μM of BH3I-2′ returned to the basal level within 700 s after application. These data show that the main source of calcium for the BH3I-2′ -induced calcium responses is in intracellular stores while external calcium plays effectively a minor role.

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_3Rs (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%).

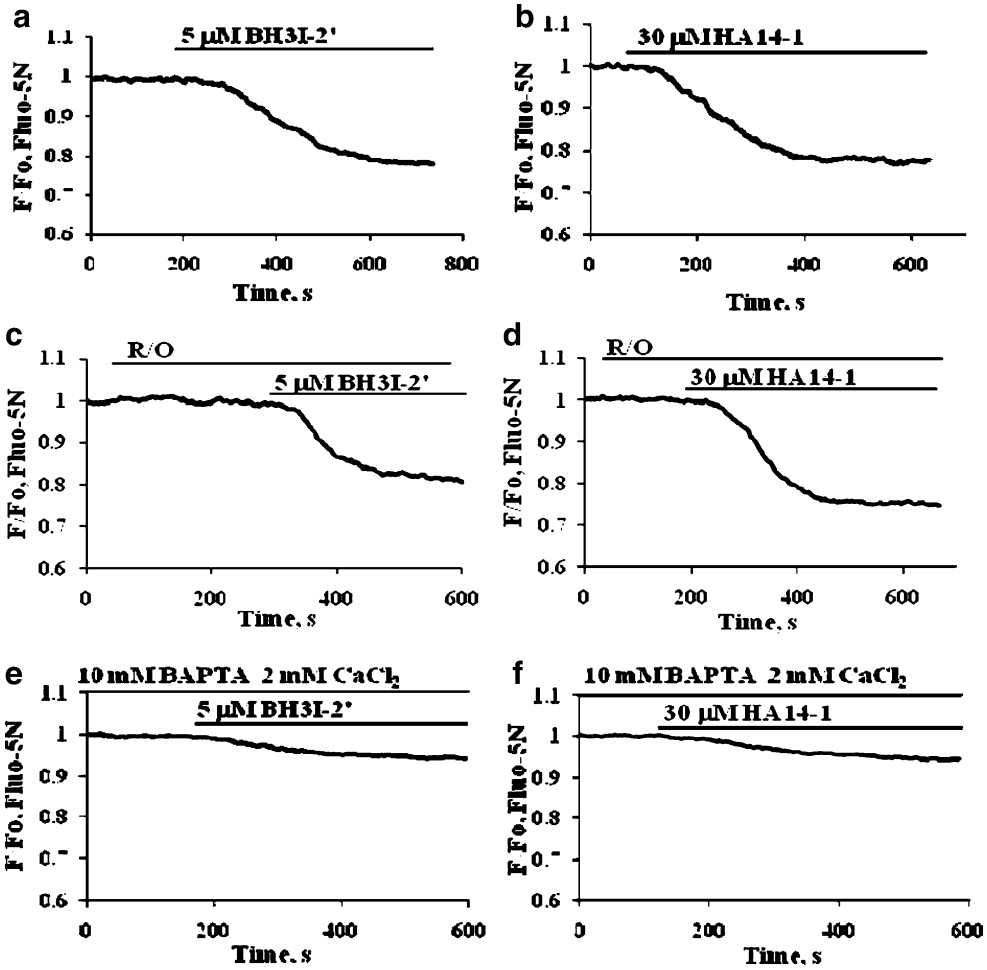

Fig. 3 Ca^{2+} release from the internal stores in response to Bcl-2/Bcl-xL inhibitors in permeabilized pancreatic acinar cells. Pancreatic acinar calcium stores were loaded with Fluo-5N and incubated in K-Hepes solution. **a** and **b** Typical trace of 5 μM BH3I-2′ (**a**)- or 30 μM HA14-1 (**b**)-elicited Ca^{2+} release from the intracellular stores. **c** and **d** Pre-treatment of permeabilized cells with mixture of 10 μM rotenone and 10 μM oligomycin did not prevent reduction of calcium content induced by both inhibitors. **e** and **f** In the condition of clamped Ca^{2+} (10 mM BAPTA/2 mM $CaCl_2$) responses of internal stores to 5 μM BH3I-2′ (**e**) or 30 μM HA14-1 (**f**) were reduced but resolvable. Pancreatic acinar cells were loaded with Fluo-5N in AM form

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 cause release of calcium from the intracellular stores and did not alter the ability of the Bcl-2/Bcl-xl inhibitors to do so.

Importantly, the investigation of the mechanism of the effect of the Bcl-2/Bcl-xl inhibitors showed that both IP_3R and RyR functions are necessary for their effects on release of calcium from intracellular stores. That is, the IP_3R 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 IP_3Rs 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

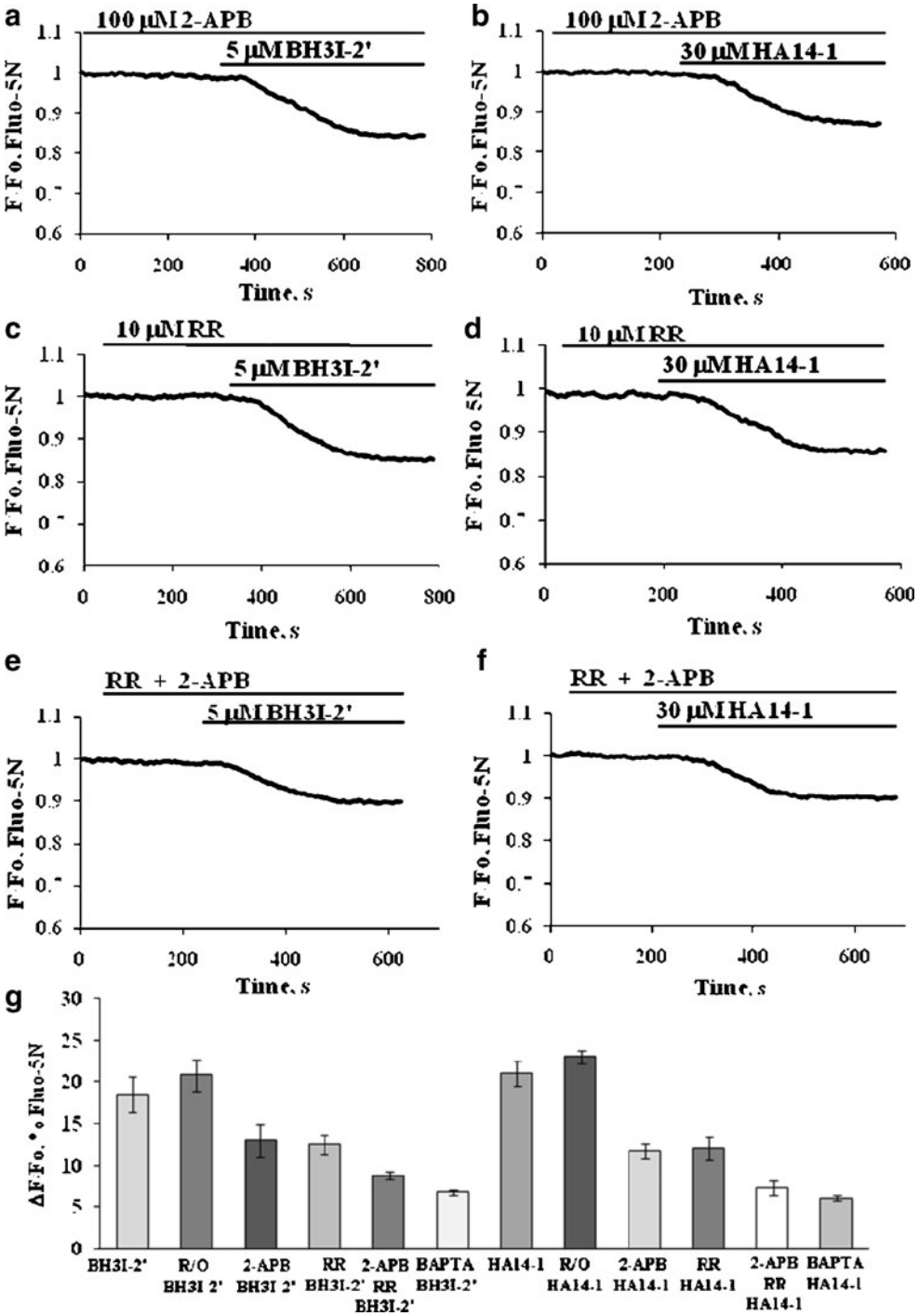

Fig. 4 Inhibition of IP_3 and RyR receptors reverses the effect of Bcl-2/Bcl-xl inhibitors in permeabilized pancreatic acinar cells. **a** and **b** Pre-incubation of permeabilized cells with 100 μM 2-APB partially reduced responses to 5 μM BH3I-2′ (**e**) or 30 μM HA14-1 (**f**). In the condition of clamped Ca^{2+} (10 mM BAPTA/2 mM $CaCl_2$) responses of internal stores to 5 μM BH3I-2′ (**e**) or 30 μM HA14-1 (**f**) were reduced but resolvable. **c** and **d** Pre-incubation of permeabilized cells with 10 μM ruthenium red partially reduced responses to 5 μM BH3I-2′ (**a**) or 30 μM HA14-1 (**b**). **e** and **f** Pre-incubation of permeabilized cells with mixture of 100 μM 2-APB and 10 μM ruthenium red substantially reduced responses to 5 μM BH3I-2′ (**c**) or 30 μM HA14-1 (**d**). **g** Summary of data obtained on permeabilized cells with addition of both inhibitors to control permeabilized cells and to cells treated with either 10 μM rotenone/10 μM oligomycin or 100 μM 2-APB or 10 μM ruthenium red or mixture 100 μM 2-APB and 10 μM ruthenium red or in the presence of 10 mM BAPTA/2 mM $CaCl_2$. Cells were loaded with Fluo-5N in AM form

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 IP_3Rs 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

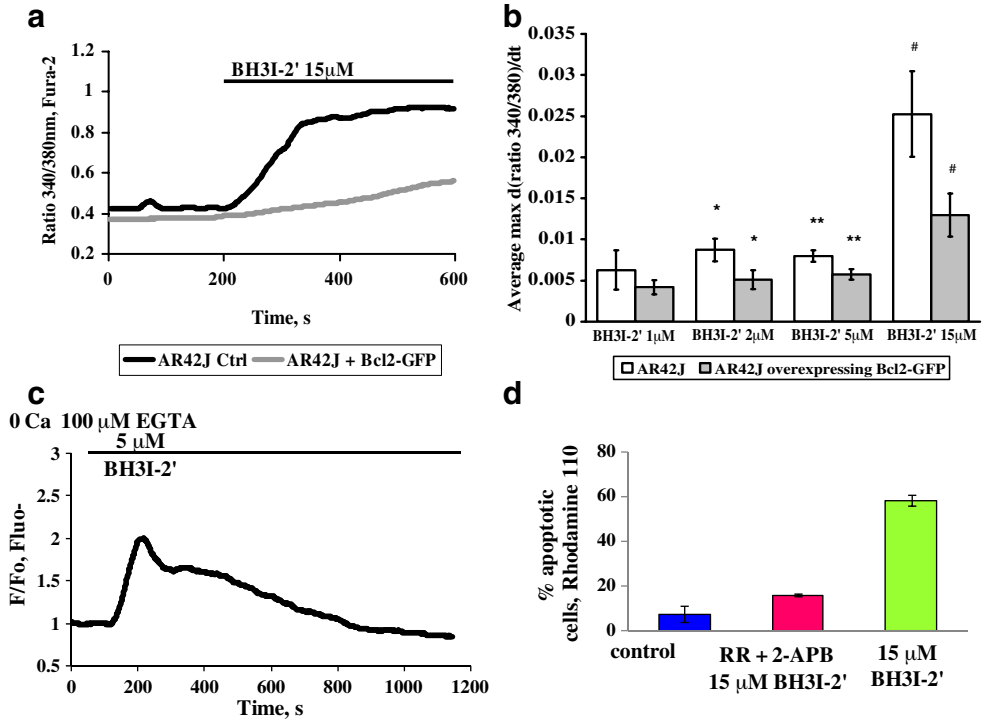

Fig. 5 CICR after application of BH3I-2′ is substantially reduced in Bcl-2 overexpressing cells. **a** Representative traces of responses to 15 μM BH3I-2′ in control (*black line*) and in Bcl-2 overexpressing AR42J cells (*grey line*). **b** Rates of the increasing phase of responses to BH3I-2′ in control (*white bars*) and Bcl-2 overexpressing AR42J cells (*grey bars*). Significant difference marked by *asterisks*, *double 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 ($n=7$). **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±3.7%), BH3I-2′-treated (15 μM) cells with (15.8±0.7%) 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 Bcl-2 family inhibitor 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 IP_3Rs and RyRs, apoptosis was dramatically reduced. Interestingly, the inhibition of IP_3Rs 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 IP_3R 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 IP_3R increasing its sensitivity to IP_3, 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 sequestrated by prosurvival Bcl-2 proteins increases the sensitivity of IP_3Rs and RyRs for activation by calcium. Considering previous studies showing that calcium activation of these receptors

(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.

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