BH3 mimetic-elicited Ca\textsuperscript{2+} signals in pancreatic acinar cells are dependent on Bax and can be reduced by Ca\textsuperscript{2+}-like peptides

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BH3 mimetics are small-molecule inhibitors of B-cell lymphoma-2 (Bcl-2) and Bcl-xL, which disrupt the heterodimerisation of anti- and pro-apoptotic Bcl-2 family members sensitising cells to apoptotic death. These compounds have been developed as anti-cancer agents to counteract increased levels of Bcl-2 proteins often present in cancer cells. Application of a chemotherapeutic drug supported with a BH3 mimetic has the potential to overcome drug resistance in cancers overexpressing anti-apoptotic Bcl-2 proteins and thus increase the success rate of the treatment. We have previously shown that the BH3 mimetics, BH3I-2\textsuperscript{′} and HA14-1, induce Ca\textsuperscript{2+} release from intracellular stores followed by a sustained elevation of the cytosolic Ca\textsuperscript{2+} concentration. Here we demonstrate that loss of Bax, but not Bcl-2 or Bak, inhibits this sustained Ca\textsuperscript{2+} elevation. What is more, in the absence of Bax, thapsigargin-elicited responses were decreased; and in two-photon-permeabilised bax\textsuperscript{−/−} cells, Ca\textsuperscript{2+} loss from the ER was reduced compared to WT cells. The Ca\textsuperscript{2+}-like peptides, CALP-1 and CALP-3, which activate EF hand motifs of Ca\textsuperscript{2+}-binding proteins, significantly reduced excessive Ca\textsuperscript{2+} signals and necrosis caused by two BH3 mimetics: BH3I-2\textsuperscript{′} and gossypol. In the presence of CALP-1, cell death was shifted from necrotic towards apoptotic, whereas CALP-3 increased the proportion of live cells. Importantly, neither of the CALPs markedly affected physiological Ca\textsuperscript{2+} signals elicited by ACh, or cholecystokinin. In conclusion, the reduction in passive ER Ca\textsuperscript{2+} leak in bax\textsuperscript{−/−} cells as well as the fact that BH3 mimetics trigger substantial Ca\textsuperscript{2+} signals by liberating Bax, indicate that Bax may regulate Ca\textsuperscript{2+} leak channels in the ER. This study also demonstrates proof-of-principle that pre-activation of EF hand Ca\textsuperscript{2+}-binding sites by CALPs can be used to ameliorate excessive Ca\textsuperscript{2+} signals caused by BH3 mimetics and shift necrotic death towards apoptosis.

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Disrupted regulation of apoptosis is the hallmark of carcinogenesis allowing accumulation of further genetic mutations and acquisition of metastatic properties.\textsuperscript{3} Cancer cells often express increased levels of anti-apoptotic Bcl-2 (B-cell lymphoma-2) proteins, which provides additional protection against cell death signals\textsuperscript{2,3} correlating with chemotherapy resistance and poor prognosis for patients.\textsuperscript{4}

As anti-cancer therapies often target the mitochondrial apoptotic pathway regulated by the Bcl-2 family, overexpression of anti-apoptotic proteins in cancer presents one of the leading challenges to overcome for effective treatment.\textsuperscript{5} Mechanism of apoptosis induction in cancer is predominantly altered upstream of Bax and Bak.\textsuperscript{6} Therefore pharmacological suppression of anti-apoptotic Bcl-2 members leading to activation of Bax and Bak should, in principle, be capable of recovering the programmed cell death.\textsuperscript{7,8}

BH3 mimetics are small-molecule synthetic inhibitors of Bcl-2 and Bcl-xL, specifically developed as anti-cancer agents.\textsuperscript{7} They mimic activated BH3-only proteins by disrupting the heterodimerisation of anti- and pro-apoptotic Bcl-2 family members, and thus sensitising cells to apoptosis. HA14-1 was the first BH3 mimetic obtained by molecular modelling that had the ability to displace Bax from Bcl-2, followed by the induction of cell death.\textsuperscript{7} Later, a family of seven members of BH3 mimetics, called BH3 inhibitors (BH3Is), was developed and shown to displace Bak peptide from Bcl-xL, trigger apoptosis, cytochrome c release and caspase activation.\textsuperscript{9} A natural BH3 mimetic gossypol, isolated from the cotton plant (Gossypium), is also capable of inhibiting Bcl-2, Bcl-xL and Mcl-1.\textsuperscript{10,11}

Simultaneous application of a chemotherapeutic agent and a BH3 mimic can potentially overcome drug resistance in cancers overexpressing anti-apoptotic Bcl-2 proteins and thus increase the treatment success rate. BH3I-2\textsuperscript{′} and HA14-1 were shown to sensitisise leukaemic cells in vitro to TRAIL-induced apoptosis.\textsuperscript{12} Gossypol (AT-101) was found to increase radiation efficacy in head and neck cancer cell lines in vitro\textsuperscript{13} and recently underwent phase II clinical trials in combination with docetaxel\textsuperscript{14} and with androgen deprivation therapy.\textsuperscript{15} A particularly interesting approach is to develop chemically tailored BH3 mimetics that interact specifically with the anti-apoptotic proteins overexpressed in a target cancer type.\textsuperscript{8}

As exciting as the prospect of Bcl-2 inhibition might seem, accumulating evidence shows that BH3 mimetics often affect intracellular Ca\textsuperscript{2+} homeostasis. Previously we have reported that HA14-1 and BH3I-2\textsuperscript{′} deplete the ER Ca\textsuperscript{2+} store causing a sustained elevated cytosolic Ca\textsuperscript{2+} concentration in pancreatic...
acinar cells (PACs). Others have demonstrated that HA14-1 causes Ca\textsuperscript{2+} deregulation in platelets. HeLa and HEK-293T cells. This immediately triggers questions about safe and specific use of BH3 mimetics, especially given the crucial role of Ca\textsuperscript{2+} in the regulation of a wide variety of intracellular process including muscle contraction, enzyme secretion, fertilisation, cell proliferation, and death. This is particularly important in the pancreas, as physiological Ca\textsuperscript{2+} oscillations control enzyme secretion in PACs, whereas abnormal Ca\textsuperscript{2+} signals are the hallmark of the initial stages of a severe necrotising disease of the pancreas – acute pancreatitis. This study aims to assess the effects of BH3 mimetics on Ca\textsuperscript{2+} handling in relation to activation of pro-apoptotic Bax in PACs. Also, we draw conclusions about the involvement of Bax in intracellular Ca\textsuperscript{2+} signalling and propose means to reduce the excessive Ca\textsuperscript{2+} signals enabling modulation of the cell death mechanisms.

Results

Ca\textsuperscript{2+} responses to BH3 mimetics in pancreatic acinar cells are dependent on Bax. BH3I-2 and HA14-1 were shown to induce a slow Ca\textsuperscript{2+} release from the intracellular stores. Pharmacological inhibition of inositol 1,4,5-triphosphate receptors (IP\textsubscript{3}Rs) and ryanodine receptors (RyRs) led to a decreased Ca\textsuperscript{2+} release from the ER of permeabilised PACs but did not completely block it. This indicates that IP\textsubscript{3}R or RyR were not the primary source of BH3 mimic-elicited Ca\textsuperscript{2+} release but merely amplified it. Here PACs, isolated from wild-type (WT) mice as well as animals with a loss-of-function mutation in one of the following genes: bcl-2, bak or bax were treated with 5 μM BH3I-2 (Figure 1a) or 30 μM HA14-1 (Figure 1b) in the absence of extracellular Ca\textsuperscript{2+}. In WT, bcl-2\textsuperscript{−/−} and bak\textsuperscript{−/−} cells, these BH3 mimetics caused sustained elevations of the cytosolic Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}), whereas in bax\textsuperscript{−/−} cells the Ca\textsuperscript{2+} signal generation was largely abolished (Figures 1a and b). Quantitative comparison of the responses to BH3I-2 (Figure 1c) and HA14-1 (Figure 1d) confirmed that loss of Bax, but not Bcl-2 or Bak, dramatically inhibited the BH3 mimic-induced elevation of the [Ca\textsuperscript{2+}]\textsubscript{i}. (P<0.001). The responses to BH3I-2 or HA14-1 in bax\textsuperscript{−/−} cells, if present at all, were mainly short-lasting Ca\textsuperscript{2+} transients, whereas sustained elevation was mostly inhibited (Figures 1e and f). Further, measurements of Ca\textsuperscript{2+} release from the ER in two-photon-permeabilised PACs revealed that BH3I-2 not only appears to release less Ca\textsuperscript{2+} from the ER of bax\textsuperscript{−/−} compared to WT cells (P<0.01; Figures 2a and b) but also that the apparent rate of release was slower than in WT cells. Consequently, t\textsubscript{1/2} for bax\textsuperscript{−/−} (226.5 ± 25.9 s) cells was higher than for WT cells (145.1 ± 19.3 s; P<0.05; Figure 2c), which translates into longer time needed for the fluorescence of the Ca\textsuperscript{2+} indicator to decrease by half the difference between baseline and final values. Images of a PAC doublet before and after laser permeabilisation are depicted in Figure 2d. Increasing the ER store loading with CDN1163, a postulated allosteric activator of SERCA2b (sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase, isoform 2b), did not significantly affect the responses to BH3I-2 in bax\textsuperscript{−/−} cells (Figure 2e). Finally, BH3I-2 increased apoptosis in WT cells by ~20.6 ± 3.8% and necrosis by 26.1 ± 2.7% over control levels; these effects were completely abolished by intracellular Ca\textsuperscript{2+} chelation with BAPTA (P<0.05; Figure 2f; bars and corresponding images). In bax\textsuperscript{−/−} cells, BH3I-2 did not affect apoptosis and only slightly increased necrosis (by 8.8 ± 3.8%); the latter was completely inhibited by BAPTA (P<0.05; Figure 2f). The effects of BH3I-2 on cell death were substantially less pronounced in bax\textsuperscript{−/−} cells compared to WT cells (P<0.05 for both apoptosis and necrosis; Figure 2f).

Loss of bax reduces Ca\textsuperscript{2+} release from the ER. The effects of Bax on intracellular Ca\textsuperscript{2+} homoeostasis were investigated in PACs that had their ER stores depleted with the SERCA blocker thapsigargin under simultaneous inhibition of IP\textsubscript{3}R (by 20 mM caffeine) and RyRs (by 10 μM ruthenium red). Average traces (Figure 3a) and average areas of responses (Figure 3a, inset) demonstrate that [Ca\textsuperscript{2+}]\textsubscript{i} elevation caused by the ER depletion was significantly smaller in bax\textsuperscript{−/−} than in WT cells (P<0.001). Inhibition of Ca\textsuperscript{2+} extrusion by 1 mM La\textsuperscript{3+} preserved the difference in size of the thapsigargin-induced Ca\textsuperscript{2+} release between WT and bax\textsuperscript{−/−} cells (P<0.01; Figure 3b) indicating that this difference was not due to enhanced cytosolic Ca\textsuperscript{2+} extrusion in bax\textsuperscript{−/−} cells. Further, in two-photon-permeabilised PACs, thapsigargin also released less Ca\textsuperscript{2+} from the ER of bax\textsuperscript{−/−} than from WT cells (P<0.05; Figures 3c and d) and the apparent rate of release was slower in bax\textsuperscript{−/−} compared to WT cells (t\textsubscript{1/2} = 278.3 ± 34.2 s versus 166.6 ± 27.7 s; P<0.05; Figure 3e). Emptying the ER store with a supramaximal dose of acetylcholine (ACh, 10 μM) resulted in only very slightly diminished responses in bax\textsuperscript{−/−} cells in the first 100 s (62.7 ± 3.7 a.u.) compared to WT cells (79.2 ± 4.4 a.u.; P<0.01; Figures 3f and g). The second emptying of the ER store, preceded by partial reloading in 1 mM extracellular Ca\textsuperscript{2+}, showed no statistically significant difference between WT (45.0 ± 2.8 a.u.) and bax\textsuperscript{−/−} cells (41.9 ± 2.0 a.u.; P=0.38; Figures 3f and g), indicating that ER store refilling was not substantially altered by loss of Bax. Finally, the responses to physiological doses of ACH (100 nM) were essentially unaffected as shown by sample traces (Figures 3h and i) and the response areas (Figure 3j).

CALPs inhibit Ca\textsuperscript{2+} entry in pancreatic acinar cells. Calcium-like peptides (CALPs) are short peptides designed by inversion of the hydrophobic pattern of EF hand Ca\textsuperscript{2+}-binding sites, which generates molecules of a complementary surface contour, capable of interacting with the sequences of interest. Cell permeable CALP-1 and CALP-3 can functionally mimic increased [Ca\textsuperscript{2+}]\textsubscript{i} by modulating the activity of calmodulin, Ca\textsuperscript{2+} channels and pumps.

Emptying the ER Ca\textsuperscript{2+} store with thapsigargin leads to opening of store-operated Ca\textsuperscript{2+} entry (SOCE) channels in the plasma membrane and influx of Ca\textsuperscript{2+} into the cytosol. As seen in Figures 4a–c, thapsigargin elicits an increase in [Ca\textsuperscript{2+}]\textsubscript{i}, which, in the absence of external Ca\textsuperscript{2+}, is transient due to the extrusion of Ca\textsuperscript{2+} by the plasma membrane Ca\textsuperscript{2+} pumps. When Ca\textsuperscript{2+} is added to the external solution, [Ca\textsuperscript{2+}]\textsubscript{i} increases again, due to SOCE (Figures 4a–c). In the absence of CALPs,
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Figure 1  Loss of Bax markedly inhibits Ca\(^{2+}\) responses induced by BH3I-2’ and HA14-1. (a) Average Ca\(^{2+}\) responses (± S.E.M.) to 5 μM BH3I-2’ in PACs isolated from WT mice (blue, n = 12), bcl-2−/− (green, n = 28), bak−/− (purple, n = 27) and bax−/− (red, n = 20). (b) Average Ca\(^{2+}\) responses (± S.E.M.) to 30 μM HA14-1 in PACs isolated from WT mice (blue, n = 26), bcl-2−/− (green, n = 23), bak−/− (purple, n = 43) and bax−/− (red, n = 17). (c) The responses shown in a were quantitatively analysed by comparing the average Ca\(^{2+}\) areas under traces recorded between 400 and 1000 s: WT (blue, n = 12, 304.6 ± 39.7 a.u.), bcl-2−/− (green, n = 28, 256.6 ± 25.5 a.u.), bak−/− (purple, n = 27, 312.0 ± 50.9 a.u.) and bax−/− (red, n = 20, 46.5 ± 11.7 a.u.). (d) The responses shown in b were quantitatively analysed by comparing the average areas under traces recorded between 400 and 1000 s: WT (blue, n = 26, 216.5 ± 35.1 a.u.), bcl-2−/− (green, n = 23, 266.6 ± 36.1 a.u.), bak−/− (purple, n = 43, 155.4 ± 22.1 a.u.) and bax−/− (red, n = 17, 47.0 ± 13.6 a.u.). (e) Patterns of Ca\(^{2+}\) responses to BH3I-2’ averaged in a. Four types were identified: (1) Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) type followed by a sustained Ca\(^{2+}\) elevation (plateau), (2) Ca\(^{2+}\) plateau with no CICR, (3) Ca\(^{2+}\) transients (CICR) with no plateau formation, (4) no response. Insets show sample traces of each identified type of response (scale: x axis: 200 s; y axis: 1.0 F/F\(_{0}\), Fluo-4). (f) Patterns of Ca\(^{2+}\) responses to HA14-1 averaged in b. The responses were classified as in e. Insets show sample traces of each identified type of response (scale: x axis: 200 s; y axis: 1.0 F/F\(_{0}\), Fluo-4).

the first and second application of 10 mM Ca\(^{2+}\) induced similar [Ca\(^{2+}\)], elevations (Figure 4a). Addition of low concentrations (10 μM) of CALP-1 (Figure 4b) or CALP-3 (Figure 4c) before the second application of 10 mM Ca\(^{2+}\) substantially inhibited Ca\(^{2+}\) influx, as shown by markedly reduced amplitudes (P<0.001; Figure 4d).

CALPs reduce excessive cytosolic Ca\(^{2+}\) signals and necrosis elicited by BH3I-2’. Excessive cytosolic Ca\(^{2+}\) signals can trigger necrosis, associated with release of intracellular content followed by inflammation.34 Necrosis is especially dangerous for the pancreas, where released activated digestive enzymes cause a serious threat for the integrity of the tissue.35,36 Therefore apoptosis, executed by cells in a controlled manner, is a more favourable pathway for killing cells. Here, CALPs were applied in an attempt to reduce elevations in [Ca\(^{2+}\)], and necrosis caused by BH3 mimetics. Preincubation of PACs either with 100 μM CALP-1 or CALP-3 led to a decrease in the average amplitude of the Ca\(^{2+}\) signals occurring in response to BH3I-2’ (P<0.001 and P<0.05, respectively) compared to the control (average traces: Figure 5a; quantitative analysis: Figure 5b). This was due to a shift in the pattern of cytosolic Ca\(^{2+}\) signals elicited by 5 μM BH3I-2’ (Figure 5c). The consequences of the CALP-mediated reductions in the cytosolic Ca\(^{2+}\) signal generation are reflected by the cell death pattern (Figures 5d and e). In the untreated control the majority of cells were alive (low levels of apoptosis and
Figure 2  Loss of Bax reduces BH3I-2'-induced Ca2+ leak from the ER and cell death in PACs. (a) Average traces (± S.E.M.) showing Ca2+ release from the ER of permeabilised WT (blue, n = 10) and bax−/− (red, n = 14) PACs induced by 5 μM BH3I-2'. Inset shows individual traces. (b) Dot chart shows individual and average (± S.E.M.) response areas below the baseline calculated between 100 and 600 s for the traces from a: WT (123.6 ± 19.7 a.u.) and bax−/− (53.3 ± 6.3 a.u.). (c) Dot chart shows the half-times (τ1/2) of the reduction in fluorescence of the Ca2+ indicator in the ER (and thus [Ca2+]ER) towards the final levels calculated for the traces depicted in a. (d) Images of a PAC doublet before and after two-photon permeabilisation. Black arrow shows the site of permeabilisation. (e) Bar chart shows average areas under traces (± S.E.M.) calculated between 200 and 1000 s for bax−/− cells pre-incubated for 2 h with 10 μM CDN1163 (grey, n = 61, 88.3 ± 9.0 a.u.) or with 0.05% DMSO (vehicle control, red, n = 89, 73.3 ± 7.1 a.u.) and then treated with 5 μM BH3I-2'. Inset shows averaged traces (± S.E.M.). (f) Apoptosis and necrosis induced by 30 min incubation with 5 μM BH3I-2' in WT and bax−/− PACs with or without 15 min pretreatment with 25 μM BAPTA. Blue bars represent apoptotic cells, and red -- necrotic. N = 3 for all the groups; individual values are indicated with grey dots. Below each pair of bars, sample images show morphology (transmitted light images) and typical annexin V (green), and propidium iodide (red) staining of PACs in the experiment.
necrosis are due to enzymatic digestion of the tissue). Incubation with 5 μM BH3I-2' resulted in a 19.1 ± 4.9% increase in apoptosis and a 31.7 ± 8.1% increase in necrosis over the control levels (similar as in Figure 2f). In the presence of CALP-1, necrosis was significantly decreased (P < 0.05), whereas apoptosis markedly increased (P < 0.05) compared to BH3I-2' alone. In contrast, CALP-3 did not affect apoptosis, but caused a reduction in necrotic cells (P < 0.05) accompanied by an increase in the proportion of live cells (P < 0.05).

**Figure 3** Loss of Bax reduces Ca2+ release from the ER. (a) Average Ca2+ responses (± S.E.M.) induced by 10 μM thapsigargin (Tg) in WT (blue, n = 9) and bax−/− (red, n = 12) PACs. IP3Rs and RyRs were inhibited by 20 mM caffeine (Caff) and 10 μM ruthenium red (RR), respectively. Inset: average areas under traces calculated between 400 and 1400 s for the individual recordings in WT (305.4 ± 15.5 a.u.) and bax−/− (168.7 ± 6.9 a.u.) cells. (b) Average Ca2+ responses (± S.E.M.) induced by 10 μM Tg in WT (blue, n = 24) and bax−/− (red, n = 21) PACs in the presence of 20 mM Caff and 10 μM RR under inhibition of Ca2+ extrusion by 1 mM La3+. Inset: average areas under traces (± S.E.M.) calculated between 400 and 1400 s for the individual recordings in WT (616.3 ± 26.5 a.u.) and bax−/− (469.4 ± 35.9 a.u.) cells. (c) Average traces (± S.E.M.) showing Ca2+ release from the ER of permeabilised WT (blue, n = 7) and bax−/− (red, n = 7) PACs induced by 2 μM Tg. Inset shows individual traces. (d) Dot chart shows individual and average (± S.E.M.) areas calculated between 100 and 600 s for the traces from c. WT (151.7 ± 25.1 a.u.) and bax−/− (83.1 ± 9.1 a.u.). (e) Dot chart shows the half-times (τ1/2) of the reduction in fluorescence of the Ca2+ indicator in the ER (and thus [Ca2+]ER) towards the final levels calculated for the traces depicted in e. (f) Average Ca2+ responses (± S.E.M.) to two applications of supramaximal doses of ACh (10 μM) in the absence of extracellular Ca2+, separated by partial reloading in 1 mM extracellular Ca2+ for 15 min. (g) Average areas under traces (± S.E.M.) calculated in the first 100 s after application of ACh for the individual recordings averaged in f. (h) 100 nM ACh induces Ca2+ oscillations in WT PACs (representative trace, n = 12). (i) 100 nM ACh induces Ca2+ oscillations in bax−/− PACs (representative trace, n = 25). (j) Dot chart shows individual and average (± S.E.M.) response areas calculated between 100 and 600 s of each recording from WT and bax−/− cells.
CALPs reduce excessive cytosolic Ca\(^{2+}\) responses and necrosis elicted by gossypol. It has been reported that gossypol is also capable of mobilising intracellular Ca\(^{2+}\),\(^{37,38}\) Similarly to BH3I-2 and HA14-1, in the absence of extracellular Ca\(^{2+}\), bax\(^{-}\) cells did not respond to gossypol (Figure 6a, black trace). In WT cells in the presence of external Ca\(^{2+}\), 20 \(\mu\)M gossypol alone caused an increase in [Ca\(^{2+}\)]\(_{\text{ER}}\), reaching a sustained elevated plateau (Figure 6a). Preincubation either with 100 \(\mu\)M CALP-1 or with CALP-3 led to a decrease in the magnitude of the Ca\(^{2+}\) responses to gossypol (Figures 6a and b; \(P<0.001\)); CALP-1 markedly decreased the proportion of cells that responded at all (Figure 6c). The effects of CALP-1 and CALP-3 on gossypol-induced cell death (Figures 6d and e) were very similar to those for BH3I-2 (Figure 5d). In the presence of CALP-1, necrosis was markedly decreased (\(P<0.05\)) and the proportion of apoptotic cells was increased (\(P<0.05\)). CALP-3 reduced gossypol-induced necrosis (\(P<0.05\)) and increased the fraction of live cells (\(P<0.05\)).

CALPs do not markedly affect physiological Ca\(^{2+}\) signals in acinar cells. The ability of CALPs to reduce excessive pathological Ca\(^{2+}\) signal generation is potentially very useful but given the crucial role of Ca\(^{2+}\) in the regulation of a wide variety of intracellular processes, it was important to test whether CALPs also affect physiological Ca\(^{2+}\) signalling. Nanomolar doses of ACh trigger enzyme secretion in PACs, whether CALPs also affect physiological Ca\(^{2+}\) signalling.\(^{24}\) Here, 50 nM ACh in acinar cells (Figure 7d). The presence of either CALP-1 (Figure 7e) or CALP-3 (Figure 7f) did not block the CCK-elicited responses, but the overall pattern was slightly changed (Figure 7g).

The effects of CALPs on cell death are not limited to BH3-mediated necrosis. As the primary mechanism by which CALPs exert their effects is the pre-activation of EF hand Ca\(^{2+}\)-binding sites on a wide variety of intracellular targets\(^{29}\) and inhibition of Ca\(^{2+}\) entry (Figures 4a–d), it is unlikely that CALP-mediated reduction in excessive Ca\(^{2+}\) signals and necrosis is limited only to the effects induced by BH3 mimetics. Menadione was previously shown to induce Ca\(^{2+}\)-dependent cell death in PACs.\(^{40}\) Here, 5 \(\mu\)M menadione was combined with a high physiological concentration of ACh (100 nM) in order to induce Ca\(^{2+}\)-dependent cell death of similar proportions of apoptosis and necrosis (25.1 ± 5.0% and 20.7 ± 1.7%, respectively; Figure 7h; sample images in Figure 7i) to those caused by BH3I-2 (Figure 5d) or gossypol (Figure 6d). In this protocol 100 \(\mu\)M CALP-1 decreased cell necrosis to control levels (\(P<0.05\)) while increasing slightly the proportions of both live and apoptotic cells; whereas 100 \(\mu\)M CALP-3 substantially reduced necrosis (\(P<0.05\)), leading to an increase in the number of live cells (\(P<0.05\)). The apoptotic fraction was essentially unaffected by CALP-3.

Discussion

It is now commonly accepted that Bcl-2 proteins are distributed in different cell compartments, not only at the outer mitochondrial membrane, but also in the cytosol, at the nuclear envelope and the ER.\(^{41–43}\) An increasing number of reports indicates that Bcl-2 itself may either directly or indirectly modulate intracellular Ca\(^{2+}\) fluxes such as: (1) Ca\(^{2+}\) release from the ER by binding to IP\(_3\)R or RyR;\(^{44–46}\) (2) Ca\(^{2+}\) reuptake into the ER by SERCA;\(^{47}\) (3) cellular Ca\(^{2+}\) extrusion by PMCA (plasma membrane Ca\(^{2+}\) ATPase);\(^{48}\) (4) and mitochondrial Ca\(^{2+}\) load.\(^{49}\)

We have previously demonstrated that the BH3 mimetics BH3I-2' and HA14-1 induce Ca\(^{2+}\) release from the intracellular stores in mouse PACs and in the rat pancreatic cancer cell line AR42J.\(^{16}\) This Ca\(^{2+}\) release was not completely blocked by inhibition of IP\(_3\)Rs and RyRs indicating involvement of other Ca\(^{2+}\) channels. We have also shown that BH3I-2' and HA14-1 displace Bax from Bcl-2 and Bcl-xL.\(^{16}\) Here we provide new insights into the mechanism of this phenomenon by demonstrating that loss of Bax, but not Bak or Bcl-2, substantially inhibited Ca\(^{2+}\) responses to BH3I-2' (Figure 1a), HA14-1 (Figure 1b) and gossypol (Figure 6a) in the absence of extracellular Ca\(^{2+}\). HA14-1 and gossypol were suggested to have off-target effects,\(^{50,51}\) and recently a novel Bcl-2 inhibitor, ABT-199, was found not to affect intracellular Ca\(^{2+}\) signalling.\(^{52}\) In our experiments not only the responses to HA14-1 and BH3I-2' were markedly inhibited in bax\(^{-}\)/− PACs (Figures 1a and b) but also BH3I-2'-elicited apoptosis was completely abolished (Figure 2f), indicating that these effects were dependent on Bax. Low levels of necrosis in bax\(^{-}\)/− cells may indeed suggest a Bax-independent killing component in the mechanism of BH3I-2' action. Importantly, BH3I-2'-induced cell death in PACs appears to be mediated by Ca\(^{2+}\) as it was blocked by chelation of intracellular Ca\(^{2+}\) with BAPTA (Figure 2f).

Our results appear to link Bax with the process of passive Ca\(^{2+}\) leak from the ER. The leak, unmasked by thapsigargin, developed more slowly in bax\(^{-}\)/− cells compared to WT cells (Figures 3c and e). Currently there is an on-going dispute about the nature of the channels mediating this process. As Bax can localise to the ER membranes\(^{53}\) and given that its structure resembles that of pore-forming bacterial toxins,\(^{54,55}\) Bax itself might be able to form a channel permeable to ions and thus contribute to the passive Ca\(^{2+}\) leak. Two out of seven \(\alpha\)-helices of Bax were suggested to function as pore-forming domains, but possibly more than one monomer of Bax would need to form a functional ion-permeable pore.\(^{56,57}\) Alternatively, as opposed to acting as a channel itself, Bax could directly or indirectly regulate the ER Ca\(^{2+}\) leak mediated by other channels. Consequently, it could be speculated that application of a BH3 mimic increases the unbound fraction of Bax, which activates Ca\(^{2+}\) channels in the ER, thus potentiating the leak (Figure 8). The leak may be further amplified by IP\(_3\)Rs and RyRs.

The reduced cytosolic Ca\(^{2+}\) release in response to thapsigargin treatment in bax\(^{-}\)/− cells could, in principle, be explained by a diminished resting ER Ca\(^{2+}\) content. However, as ACh-induced oscillations were essentially unaffected in bax\(^{-}\)/− cells (Figures 3h–j), this may indicate that the [Ca\(^{2+}\)]\(_{\text{ER}}\)
extracellular Ca²⁺ triggered Ca²⁺ entry into the cytosol. The first response to 10 mM Ca²⁺ was an internal control; the second response was inhibited in the presence of 10 μM CALP-1 (sample trace, n = 19); control experiment for b and c. The ER store was emptied with Tg in the absence of extracellular Ca²⁺. Application of 10 mM extracellular Ca²⁺ triggered Ca²⁺ entry into the cytosol. The first response to 10 mM Ca²⁺ was an internal control; the second response was inhibited in the presence of 10 μM CALP-1 (sample trace, n = 19). (c) The ER store was emptied with Tg in the absence of extracellular Ca²⁺. Application of 10 mM extracellular Ca²⁺ triggered Ca²⁺ entry into the cytosol. The first response to 10 mM Ca²⁺ was an internal control; the second response was inhibited in the presence of 10 μM CALP-3 (sample trace, n = 7). (d) The chart shows amplitudes (individual points and mean ± S.E.M.) of the second Ca²⁺ entry response calculated in control experiments (0.346 ± 0.027 a.u., n = 20) and in the presence of 10 μM CALP-1 (0.075 ± 0.004 a.u., n = 20) or 10 μM CALP-3 (0.046 ± 0.003 a.u., n = 7).

Figure 4  CALPs inhibit Ca²⁺ entry in PACs. (a) The ER store was emptied with thapsigargin (Tg) in the absence of extracellular Ca²⁺. Two consecutive applications of 10 mM extracellular Ca²⁺ triggered Ca²⁺ entry into the cytosol (sample trace, n = 19); control experiment for b and c. (b) The ER store was emptied with Tg in the absence of extracellular Ca²⁺. Application of 10 mM extracellular Ca²⁺ triggered Ca²⁺ entry into the cytosol. The first response to 10 mM Ca²⁺ was an internal control; the second response was inhibited in the presence of 10 μM CALP-1 (sample trace, n = 19). (c) The ER store was emptied with Tg in the absence of extracellular Ca²⁺. Application of 10 mM extracellular Ca²⁺ triggered Ca²⁺ entry into the cytosol. The first response to 10 mM Ca²⁺ was an internal control; the second response was inhibited in the presence of 10 μM CALP-3 (sample trace, n = 7). (d) The chart shows amplitudes (individual points and mean ± S.E.M.) of the second Ca²⁺ entry response calculated in control experiments (0.346 ± 0.027 a.u., n = 20) and in the presence of 10 μM CALP-1 (0.075 ± 0.004 a.u., n = 20) or 10 μM CALP-3 (0.046 ± 0.003 a.u., n = 7).

has not been substantially affected by loss of Bax. Previous experiments demonstrated that ACh-evoked short-lasting Ca²⁺ spikes in PACs are very sensitive to even minor reductions in [Ca²⁺]ER and cease after a relatively modest decrease in [Ca²⁺]ER. This could shed new light on a seeming discrepancy found in the previous studies, whereby both knockdown and overexpression of Bax led to a reduction in Ca²⁺ responses induced by various agonists or inhibition of SERCA. It is likely that upon loss of Bax, some of the leak channels become inactive, and those that remain active may be sensitive to changes in [Ca²⁺]ER and, for example, close early in the release period thereby limiting Ca²⁺ release from the ER. In contrast, overexpression of Bax may lead to an increased basal leak, which results in a decrease in resting [Ca²⁺]ER and thus dampens Ca²⁺ responses. Finally, under normal conditions, Bax is sequestered by anti-apoptotic Bcl-2 family proteins. But any shifts in the balance between anti- and pro-apoptotic Bcl-2 members may promote mechanisms that either favour pro-survival Ca²⁺ transients or larger pro-apoptotic Ca²⁺ signals.

Further, our results show that the BH3 mimetics, BH3I-2 and gossypol not only induce apoptosis in PACs but also cause substantial levels of necrosis (Figures 5d and 6d). Cell death in PACs was a downstream effect of large cytosolic Ca²⁺ signals triggered by application of BH3 mimetics, as (1) Ca²⁺ chelation by BAPTA completely abolished both apoptosis and necrosis induced by BH3I-2 and (2) reduction of Ca²⁺ signals elicited by CALP-1 or CALP-3 (Figures 5a and 6a) led to a significant decrease in BH3I-2- as well as gossypol-induced necrosis (Figures 5d and 6d). CALP-1 was shown to be particularly promising, as it effectively shifted the cell death mode from necrosis to apoptosis. In contrast, CALP-3 substantially reduced BH3 mimetic-induced necrosis while increasing the fraction of live cells. Both CALPs were capable of inhibiting Ca²⁺ entry (Figures 4a–d), even though they differ in structure. CALP-1 has been designed to interact with EF hand Ca²⁺-binding sites of troponin C, whereas CALP-3 is complementary to the EF hand motif of calmodulin. It is therefore likely that the two CALPs might preferentially interact with different intracellular targets, for example, Ca²⁺ channels regulated by Ca²⁺ versus calmodulin, abundant in PACs. This could explain different effects on BH3 mimetic-induced cell death. Previously we demonstrated that CALP-3 efficiently inhibited Ca²⁺ responses induced by ethanol in PACs and we attributed this effect to activation of calmodulin. The exact assessment of binding affinities of CALPs to intracellular targets exceeds the scope of this work. It is clear, however, that the effects of CALPs on cell death are not limited to BH3 mimetics, as necrosis triggered by ACh and menadione was also inhibited in the presence of CALPs (Figure 7h).

Considering the vast spectrum of targets affected by CALPs, it was important to test whether application of these compounds would affect not only pathological Ca²⁺ elevations but also physiological Ca²⁺ signals. In PACs, nanomolar doses of ACh and picomolar concentrations of CCK are known to trigger Ca²⁺ oscillations, which regulate pancreatic enzyme secretion. In our experiments neither CALP-1 nor CALP-3 markedly affected responses to ACh (Figures 7a–c), whereas in the presence of CALP-3, CCK-elicited Ca²⁺ oscillations were slightly reduced but not
completely inhibited (Figures 7d–g). The previously demonstrated inhibition of Ca\(^{2+}\) influx into PACs by the CRAC channel inhibitor GSK-7975A also affected only pathological Ca\(^{2+}\) elevations but not the oscillations induced by physiological concentrations of ACh or CCK.

Although it can be triggered by sustained elevations of [Ca\(^{2+}\)], necrosis generally lacks intracellular regulatory mechanisms. This severely limits possible therapeutic approaches in diseases where necrosis plays a major role, such as acute pancreatitis. Inhibition of SOCE has already been demonstrated to decrease cell death in vivo\(^66\) and in vivo.\(^67\) This study provides another proof-of-principle demonstration that even non-specific attenuation of intracellular Ca\(^{2+}\) fluxes can dampen pathophysiological Ca\(^{2+}\) responses and thus result in necrosis inhibition (CALP-3) or a significant shift in cell death towards apoptosis (CALP-1).

Our results indicate that application of CALPs, especially CALP-1, could improve the outcome of BH3 mimetic-based therapies. Although the pharmacokinetic properties of CALPs have not yet been studied in detail, it is likely that CALPs share characteristics of other short peptides, such as limited cell permeability and metabolic instability.\(^68\) However,
development of synthetic non-peptide compounds activating EF hand Ca$^{2+}$-binding motifs could potentially overcome these limitations, providing a useful pharmacological switch of cell death mode, particularly in cancer therapies.

Materials and Methods

**Reagents.** The main reagents for cell isolation and imaging include: Fluo-4 AM, Fura-2 AM, Fluor-5N AM and BAPTA AM (ThermoFisher Scientific, Paisley, UK); collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA); inorganic salts (Sigma-Aldrich, Gillingham, UK); other reagents: HA14-1 (Alexis Biochemicals, San Diego, CA, USA); BH3I-2, gossypol (Santa Cruz Biotechnology, Dallas, TX, USA); caffeine and thapsigargin (Calbiochem, Nottingham, UK); CALP-1, CALP-3, ruthenium red and CDN1163 (Tocris Bioscience, Bristol, UK). NaHHEPES buffer was prepared as follows (mM): NaCl 140, KCl 15, glucose 10; pH 7.2. KHEPES (intracellular solution) consisted of (mM): KCl 130, HEPES 10, MgCl$_2$ 1, glucose 10; pH 7.2. KHEPES (intracellular solution) consisted of (mM): KCl 130, NaCl 18, MgCl$_2$ 1, HEPES 10, ATP 3, EGTA 0.1, CaCl$_2$ 0.05; pH 7.2.

**Animals.** All procedures involving animals were performed in accordance with the UK Home Office regulations. C57BL/6J mice (male, 6-8 weeks old, 23 ± 3 g weight) were supplied by Charles River Laboratories (Margate, UK); transgenic mice bcl-2$^{-/-}$ (B6;129S2-Bcl2tm1Flv/J), bax$^{-/-}$ (B6.129X1-Bak1tm1Thsn/J) were obtained from The Jackson Laboratories (Bar Harbor, ME, USA); bax$^{-/-}$/bak$^{-/-}$ (B6.129-Bak1tm1Thsn/J) was a gift of Professor David Mark Pritchard (University of Liverpool); bax$^{-/-}$/bak$^{-/-}$ strain extensively crossed into the CD1 background was also obtained from Professor Alun Davies (Cardiff University). Transgenic mice were bred in house either from null (bax$^{-/-}$) or heterozygous (bcl-2$^{-/-}$, bax$^{-/-}$) parents; the genotype of each mouse was confirmed by PCR reaction with

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**Figure 6** CALPs reduce Ca$^{2+}$ responses and cell death induced by gossypol. (a) Average Ca$^{2+}$ responses (± S.E.M.) in PACs to 20 μM gossypol (blue, n = 20) and to gossypol in the presence of 100 μM CALP-1 (red, n = 15) or CALP-3 (green, n = 14); black trace shows lack of responses to 20 μM gossypol in bax$^{-/-}$ PACs in the absence of extracellular Ca$^{2+}$ (n = 3). (b) The responses shown in (a) were quantitatively analysed by comparing the average areas under traces recorded between 200 and 1000 s: control (blue, n = 20, 721.1 ± 79.4 a.u.), CALP-1 (red, n = 15, 213.4 ± 55.1 a.u.) and CALP-3 (green, n = 14, 320.6 ± 29.6 a.u.). (c) Distribution of different types of Ca$^{2+}$ responses (averaged in a) induced in PSCs by 20 μM gossypol in the presence/absence of 100 μM CALP-1 or CALP-3. Insets show sample traces of different response patterns; colours and scale values (x axis: 200 s; y axis: 0.5 R/R$_0$, Fura-2) correspond with those shown in a. 65% of the cells responded immediately, whereas responses in 35% of cells were delayed. CALP-1 decreased the proportion of cells that responded to gossypol either immediately (33%) or with a delay (27%) and as many as 40% of cells did not show any [Ca$^{2+}$]$_i$ elevation. In contrast, CALP-3 did not affect the pattern of the responses to gossypol – all cells developed either immediate (64%) or delayed (36%) elevations in [Ca$^{2+}$]$_i$, but of a lower amplitude than in cells treated with gossypol alone (shown in a). (d) Apoptosis and necrosis induced by 30 min incubation with 5 μM gossypol in PACs after 15 min pretreatment with 100 μM CALP-1 or CALP-3. Grey bars represent live cells, blue – apoptotic cells and red – necrotic. N = 3 for all the groups; individual values are shown with different markers (O, ▲, ▼). (e) Sample images show typical annexin V (green) and propidium iodide (red) staining of PACs used in the experiment shown in d.
Figure 7  CALPs do not inhibit physiological Ca\(^{2+}\) responses in PACs. (a) 50 nM ACh induces Ca\(^{2+}\) oscillations in PACs (representative trace, \(n = 15\)). (b) 50 nM ACh induces Ca\(^{2+}\) oscillations in PACs in the presence of 100 \(\mu\)M CALP-1 (representative trace, \(n = 12\)). CALP-1 was present 200 s before the addition of ACh. (c) 50 nM ACh induces Ca\(^{2+}\) oscillations in PACs in the presence of 100 \(\mu\)M CALP-3 (representative trace, \(n = 10\)). CALP-3 was present 200 s before the addition of ACh. (d) Typical large Ca\(^{2+}\) transients (upper trace), mixed responses (middle trace) and small oscillations (lower trace) induced by 5 pM CCK in PACs (representative traces, \(n = 9\)). Black arrow indicates addition of CCK. (e) Typical large Ca\(^{2+}\) transients (upper trace), mixed responses (middle trace) and small oscillations (lower trace) induced by 5 pM CCK in PACs in the presence of 100 \(\mu\)M CALP-1 (representative traces, \(n = 15\)). CALP-1 was present 200 s before the addition of CCK. (f) Typical large Ca\(^{2+}\) transients (upper trace), mixed responses (middle trace) and small oscillations (lower trace) induced by 5 pM CCK in PACs in the presence of 100 \(\mu\)M CALP-3 (representative traces, \(n = 11\)). CALP-3 was present 200 s before the addition of CCK. (g) Distribution of different types of Ca\(^{2+}\) responses (representative traces shown in (d-f)) induced by 5 pM CCK in the presence/absence of 100 \(\mu\)M CALP-1 or 100 \(\mu\)M CALP-3. The majority of the control responses (56%) were large transients, 22% were small oscillations and the remaining 22% consisted of both large and small Ca\(^{2+}\) spikes. Preincubation with 100 \(\mu\)M CALP-1 led to a slight decrease in the proportion of cells that responded with large Ca\(^{2+}\) oscillations (47%) accompanied by an increase in small responses (33%). CALP-3 reduced large transients even further (18%) resulting in higher proportions of cells responding with both large and small (45%), or small oscillations only (36%). (h) Apoptosis and necrosis induced by 30 min incubation with 5 \(\mu\)M menadione (Men) and 100 nM ACh in PACs after 15 min pretreatment with 100 \(\mu\)M CALP-1 or CALP-3. Grey bars represent live cells, blue – apoptotic cells and red – necrotic. \(N = 3\) for all the groups; individual values are shown with different markers (○ ▲ □). (i) Sample images show typical annexin V (green) and propidium iodide (red) staining of PACs used in the experiment shown in (h).
Isolation of pancreatic acinar cells. PAC isolation and most of the experimental work was carried out in NaHEPES buffer. Unless otherwise stated, NaHEPES was supplemented with 1 mM Ca\(^{2+}\). Freshly isolated pancreas was washed twice in NaHEPES, injected with collagenase (200 µ/ml, in NaHEPES) and subsequently incubated at 37 °C for 15 min in the collagenase solution to allow digestion of the tissue. After incubation, the pancreas was broken down by pipetting, suspended in NaHEPES, spun (1 min, 0.2 × g), resuspended in NaHEPES and spun again. Finally, isolated PACs were suspended in NaHEPES and loaded with a Ca\(^{2+}\) sensitive dye as described below.

Cytosolic Ca\(^{2+}\) measurements. Isolated PACs were loaded at room temperature with one of the Ca\(^{2+}\) indicators: 5 µM Fluo-4 AM for 30 min or 10 µM Fura-2 AM for 1 h. After the incubation the cells were resuspended in fresh NaHEPES and used for experiments at room temperature in a flow chamber perfused with NaHEPES-based extracellular solution. Experiments with Fluo-4 AM were performed using the Leica confocal microscope TCS SPE (Leica Microsystems, Milton Keynes, UK): x63 oil objective, excitation 488 nm, emission 500–600 nm. Static images were taken at 512 x 512 pixel resolution and series of images were recorded at 256 x 256 pixel resolution, two consecutive frames were averaged. Fluorescence signals were plotted as F/F\(_0\), where F\(_0\) was an averaged signal from the first ten baseline images. Experiments with Fura-2 AM were performed using the Nikon Diaphot 200 imaging system (Nikon, Kingston, UK): excitation at 365 and 385 nm, emission at 510 nm. The signals were plotted as 365/385 nm ratio or the ratio normalized to baseline values (R/R\(_0\)).

ER Ca\(^{2+}\) measurements. PACs were loaded with 5 µM Fluo-5N AM in NaHEPES for 45 min at 37 °C. After the incubation, the cells were resuspended in fresh NaHEPES and used for experiments at room temperature in a flow chamber perfused with KHEPES-based intracellular solution. The cells were permeabilised with two-photon laser beam (720–750 nm), which was applied at a small area of the plasma membrane of a PAC. The experiments were performed using the Leica two-photon confocal microscope TCS SP5 with the same settings as for Fluo-4 AM (see above).

Cell death assay. Cell death assay was performed using Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) according to a modified manufacturer’s protocol. PACs were isolated as described above and divided equally into four experimental groups. Two samples were pretreated with 100 µM CALP-1 or 100 µM CALP-3 for 15 min at room temperature; the remaining two were incubated without pretreatment. Then cell death was induced for 30 min in both samples containing CALPs and in one untreated sample. Depending on the experiment, either one of BH3 mimetics was used (5 µM BH3I-2 or 5 µM gossypol) or 5 µM menadione together with 100 nM ACh. Analogical experiments were performed with 15 min pretreatment with BAPTA instead of CALPs. The control samples were left untreated. Fifteen minutes before the end of the incubation, annexin V-FITC and propidium iodide were added to all samples. The cells were visualised with the Leica confocal microscope TCS SPE. Annexin V-FITC specifically stained apoptotic cells (excitation: 488 nm, emission: 510–570 nm), whereas propidium iodide was used...
for detection of necrotic cells (excitation: 535 nm, emission: 585–705 nm). Multiple pictures (20–35) per treatment group were taken; live, apoptotic and necrotic cells were counted in each treatment group.

Statistical analysis. For quantitative analysis of Ca2+ responses, areas under individual traces were calculated according to the formula: \[ \Sigma (F_{t} - F_{0}) \times \Delta t, \] where \( F \) is the recorded fluorescence (or ratio for Fura-2), \( F_{0} \) is the baseline fluorescence (or ratio) and \( \Delta t \) = time interval. Obtained values were then averaged and presented as bar charts with S.E.M. The Student’s t-test was applied for statistical comparison. The significance threshold was set at 0.05 and the range was indicated by asterisks (* \( p < 0.05 \)), ** \( p < 0.01 \), *** \( p < 0.001 \). Where applicable, \( N \) indicates the number of individual experiments, whereas \( n \) – individual cells.

For cell death assays, three independent experiments were performed for each treatment group; average values and S.E.M. were calculated and the results presented as bar charts. Statistical analysis was performed using the non-parametric Mann–Whitney U-test with the significance threshold set at 0.05.

Conflict of Interest
The authors declare no conflict of interest.

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1. Delbridge AR, Grabin V, Wsringer JJ, et al. Identification of the NADPH oxidase complex in nuclear membranes of human cancer cells. J Cell Sci 2016; 16: 101–110.
2. Vogler M, Dinsdale D, Ouy J, Cohen GM. Cbl-b inhibitors: small molecules with a big impact on cancer therapy. Cell Death Diff 2009; 16: 360–367.
3. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 2007; 26: 1324–1337.
4. Miyashita T, Reed JC. Bcl-2 oncprotein blocks chemotherapy-induced apoptosis in a human melanoma cell line. Blood 1993; 81: 151–157.
5. Czabotar PE, Lessene G, Adams JM. Control of apoptosis by the BCL-2 family of proteins. Cell Mol Life Sci 2014; 15: 49–63.
6. Hanahan D, Weinberg RA. The hallmarks of cancer. Nat Rev Cancer 2000; 6: 924–944.
7. Gossypol induces Bax/Bak-independent apoptosis in human melanoma cells. J Biol Chem 2004; 279: 2685–2690.
8. Jan CR, Lin MC, Chou KJ, Huang JK. Novel effects of gossypol, a chemical contraceptive in male: mobilization of internal Ca2+ and activation of external Ca2+ entry in intact cells. Biochim Biophys Acta 2000; 1496: 270–276.
9. Cheng JS, Liu CP, Lo YK, Chou KJ, Lin MC, Su W et al. Gossypol, a component in cottonseed, induces increases in cytosolic Ca2+ levels in Chang liver cells. Toxicol 2002; 60: 851–866.
10. Maran MK, Su Z, Vilain M, Blaouzic J, et al. A new type of Ca2+ channel blocker that targets Ca2+ sensors and prevents Ca2+-mediated apoptosis. FASEB J 2000; 14: 1297–1306.
11. Parekh AB. On the activation mechanism of store-operated calcium channels. Pflugers Archiv 2000; 453: 303–311.
12. Cridde DN, Gerasimenko JV, Baumgärtner HK, Farfalla M, Neoptolemos JP et al. Calcium signaling and pancreatic cell death: apoptosis or necrosis? Cell Death Diff 2007; 14: 1295–1304.
13. Petersen OH, Sutton R. Ca2+ signalling and pancreatitis: effects of alcohol, bile and coffee. Trends Pharmacol Sci 2006; 27: 113–120.
14. Gerasimenko JV, Gerasimenko OV, Petersen OH. The role of Ca2+ in the pathophysiology of pancreatitis. J Physiol 2014; 592: 269–280.
15. Murphy KL, Gerasimenko JV, Thorne C, Ferdek P, Pozzan T, Tepikin AV et al. Cell Calcium elevation in mitochondria is the main Ca2+ requirement for mitochondrial permeability transition pore (mPTP) opening. J Biol Chem 2009; 284: 20796–20803.
16. Hockenberry D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. Nature 1990; 348: 334–336.
17. Krajewski S, Tanaka S, Takayama S, Neoptolemos JP, Sutton R et al. Calcium-dependent enzyme activation and vacuole formation in the apical granular region of pancreatic acinar cells. J Cell Sci 2002; 115: 1326–1331.
18. Baumgärtner HK, Gerasimenko JV, Thorne C, Ferdek P, Pozzan T, Tepikin AV et al. Cell Calcium elevation in mitochondria is the main Ca2+ requirement for mitochondrial permeability transition pore (mPTP) opening. J Biol Chem 2009; 284: 20796–20803.
19. Chen R, Valenzuela I, Zong F, McColl KS, Roderick HL, Bootman MD et al. Bcl-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 2004; 164: 192–203.
20. Rong YP, Byuncky G, Aromolban AS, Zong F, Parry JB, De Smedt H et al. The Bih4 domain of Bcl-2 inhibits ER calcium release and apoptosis by binding to the regulatory and coupling domain of the IP3 receptor. Proc Natl Acad Sci USA 2006; 103: 14397–14402.
21. Venkataraju BV, Smith GM, Klemm WW, et al. Bcl-2 binds to and inhibits mammalian TRPM7 channels. J Cell Sci 2014; 127(Pt 12): 2758–2772.
22. Kuo TH, Kim HR, Zhu L, Yu L, Lin HM, Tsang W. Modulation of endoplasmic reticulum calcium pump by Bcl-2. Oncogene 1998; 17: 1903–1910.
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