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Involvement of ryanodine-operated channels in *tert*-butylhydroperoxide-evoked Ca^{2+} mobilisation in pancreatic acinar cells

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Summary

Reactive oxygen species and related oxidative damage have been implicated in the initiation of acute pancreatitis, a disease characterised in its earliest stages by disruption of intracellular Ca^{2+} homeostasis. The present study was carried out in order to establish the effect of the organic pro-oxidant, *tert*-butylhydroperoxide (*t*BHP), on the mobilisation of intracellular Ca^{2+} stores in isolated rat pancreatic acinar cells and the mechanisms underlying this effect. Cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_c$) were monitored using a digital microspectrofluorimetric system in fura-2 loaded cells. In the presence of normal extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$), perfusion of pancreatic acinar cells with 1 mmol l^{-1} *t*BHP caused a slow sustained increase in $[Ca^{2+}]_c$. This increase was also observed in a nominally Ca^{2+}-free medium, indicating a release of Ca^{2+} from intracellular stores. Pretreatment of cells with *t*BHP abolished the typical Ca^{2+} response of both the physiological agonist CCK-8 (1 nmol l^{-1}) and thapsigargin (TPS, 1 μmol l^{-1}), an inhibitor of the SERCA pump, in the absence of extracellular Ca^{2+}. Similar results were observed with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, 0.5 μmol l^{-1}), a mitochondrial uncoupler. In addition, depletion of either agonist-sensitive Ca^{2+} pools by CCK-8 or TPS or mitochondrial Ca^{2+} pools by FCCP were unable to prevent the *t*BHP-induced Ca^{2+} release. By contrast, simultaneous administration of TPS and FCCP clearly abolished the *t*BHP-induced Ca^{2+} release. These results show that *t*BHP releases Ca^{2+} from agonist-sensitive intracellular stores and from mitochondria. On the other hand, simultaneous application of FCCP and of 2-aminoethoxydiphenylborane (2-APB), a blocker of IP_3-mediated Ca^{2+} release, was unable to suppress the increase in $[Ca^{2+}]_c$ induced by *t*BHP, while the application of 50 μmol l^{-1} of ryanodine (which is able to block the ryanodine channels) inhibits *t*BHP-evoked Ca^{2+} mobilisation. These findings indicate that *t*BHP releases Ca^{2+} from non-mitochondrial Ca^{2+} pools through ryanodine channels.

Key words: *tert*-butylhydroperoxide (*t*BHP), cytosolic Ca^{2+}, reactive oxygen species, pancreatic acinar cell, mitochondria, endoplasmic reticulum (ER), IP_3 (inositol-1,4,5-triphosphate), ryanodine channel.

Introduction

Calcium ions (Ca^{2+}) are a universal intracellular messenger, controlling a diverse range of cellular processes in birth, life and death, such as gene transcriptional activation, contraction and secretion, or cell differentiation and proliferation (Carafoli et al., 2001). Ca^{2+} concentration in the cytosolic environment changes in response to a variety of simultaneous signals, which differ in their origin (extra- or intracellular). In most cells, Ca^{2+} has a major signalling function when its concentration is elevated in the cytosolic compartment ($[Ca^{2+}]_c$) (Berridge et al., 2000).

$[Ca^{2+}]_c$ elevation has been mainly attributed to: (i) entry of external Ca^{2+} through plasma membrane channels (Putney, 1988); (ii) Ca^{2+} release from intracellular Ca^{2+} agonist-sensitive stores, which might be mediated by either inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Moreau et al., 1998) or by activation of several distinct types of messenger-activated channels [e.g. inositol-1,4,5-triphosphate (IP_3)- and ryanodine-operated channels]. Additionally, mitochondria can trigger and perpetuate cytosolic Ca^{2+} signals *via* mitochondrial permeability transition activation (Duchen, 2000), contributing to Ca^{2+}-induced Ca^{2+}-release (CICR) (González and Salido, 2001). $[Ca^{2+}]_c$ is returned to basal levels by: (i) Ca^{2+} extrusion through plasma membrane pumps and exchangers (Camello et al., 1996) and (ii) Ca^{2+} reuptake into cytosolic and mitochondrial pools (Tepikin et al., 1992).

Reactive oxygen species (ROS) can be used as messengers in normal cell functions (Rosado et al., 2004). However, at oxidative stress levels they can disrupt physiological pathways and cause cell death. In addition, it has been shown that intracellular Ca^{2+} appears to play a role as a signal transducer in the mechanism of apoptosis (Distelhorst and Dubyak, 1998). The effects of oxidants on Ca^{2+} signalling can vary from stimulatory to repressive, depending on the type of oxidants, their concentrations, and the duration of the exposure (Waring, 2005). However, it is generally reported that oxidants can cause a rapid increase in $[Ca^{2+}]_c$ in diverse cell types (Rooney et al., 1991; Wang and Joseph, 2000), which can precede other morphological and functional alterations. Oxidants can also regulate the production of IP_3 and Ca^{2+} release from the endoplasmic reticulum/sarcoplasmic reticulum (ER/SR) (Doan et al., 1994). SERCA can be inhibited both by oxidation of its sulphydryl groups and by direct attack of oxidants on the ATP binding site (Castilho et al., 1996; Redondo et al., 2004). Plasma membrane ATPases are also inhibited by oxidants (Zaidi et al., 2003; Redondo et al., 2004). In addition, previous studies have evaluated the effect of free radicals generated by xanthine oxidase-catalyzed oxidation of hypoxanthine on the cellular function of isolated rat pancreatic acinar cells, showing a rapid and sustained increase in $[Ca^{2+}]_c$ (Klonowski-Stumpe et al., 1997).

Another known potent oxidant, menadione, evokes repetitive cytosolic Ca^{2+} spikes, partial mitochondrial depolarisation, cytochrome *c* release and apoptosis in isolated pancreatic acinar cells (Gerasimenko et al., 2002). Studies in our laboratories show that treatment of rat pancreatic acinar cells with hydrogen peroxide (H_2O_2) results in the release of Ca^{2+} from mitochondrial and non-mitochondrial intracellular Ca^{2+} stores, and this action is mediated by oxidation of sulphydryl groups of Ca^{2+}-ATPases (Pariente et al., 2001). Additionally, H_2O_2 can evoke marked changes in mitochondrial activity that might be due to the oxidant nature of H_2O_2 (González et al., 2005).

*t*BHP is a prototypical organic pro-oxidant and has been used to study the role of Ca^{2+} in oxidant-induced cell death (Jones et al., 1983; Liu et al., 1998). *t*BHP is an inducer of apoptosis and cellular damage through oxidative stress (Gorbunov et al., 1998). It has been previously shown that *t*BHP decreases the cell membrane resistance, triggering apoptosis (Lang et al., 2003), and induces lipid peroxidation and malondialdehyde formation (Rush et al., 1985), mobilising arachidonic acid from membrane phospholipids through a phospholipase A(2)-mediated mechanism (Martín et al., 2001; Masaki et al., 1989). Additionally, *t*BHP inhibits the plasma membrane Ca^{2+}-pump ATPase (PMCA) (Rohn et al., 1993), and enhances mitochondrial Ca^{2+} uptake, leading to increased matrix Ca^{2+} levels and onset of the permeability transition pore (Byrne et al., 1999).

The effects of *t*BHP on Ca^{2+} mobilisation in exocrine pancreas, however, have only been investigated in a few studies. In rat pancreatic acinar cells, *t*BHP disrupts repetitive Ca^{2+} spiking in response to carbachol, leading to a sustained increase in $[Ca^{2+}]_c$ (Sweiry et al., 1999). Nevertheless, the intracellular mechanisms underlying these effects remain unclear. Thus, the aim of the present study was to investigate the effect of *t*BHP on $[Ca^{2+}]_c$ in collagenase-dispersed rat pancreatic acinar cells and to study the mechanisms involved, using an epifluorescence inverted microscope.

Materials and methods

Animals and chemicals

Adult male Wistar rats *Rattus norvegicus albinus* Berkenhaut (mass 120–150 g) were used throughout this study and obtained from the Animal Farm of the Faculty of Veterinary Sciences, University of Extremadura (Cáceres, Spain). Fura-2/AM was purchased from Molecular Probes Europe (Leiden, Netherlands). Collagenase CLSPA was obtained from Worthington Biochemical Corporation (Freehold, NJ, USA) and TPS from Alomone Labs (Jerusalem, Israel). All other reagents used were obtained from Sigma (Madrid, Spain).

Preparation of isolated rat pancreatic acinar cells

A suspension of single cells and small acini was obtained from isolate rat pancreas as described previously (Martínez et al., 2004). Briefly, after cervical dislocation of animals, the pancreas was rapidly removed, treated by enzymatic digestion with collagenase (Worthington, 40 U ml^{-1}) and incubated at 37°C under gentle agitation. This enzymatic digestion was followed by mechanical dispersion, by gently pipetting the cell suspension. Acinar cells were suspended in a physiological salt solution (Na-Hepes buffer) containing: 0.1 mg ml^{-1} soybean trypsin inhibitor, 0.2% (w/v) bovine serum albumin and (in mmol l^{-1}): 140 NaCl, KCl 4.7, $MgCl_2$ 1.1, *N*-2-hydroxyethylpiperazine-*N*′-2-sulphonic acid (Hepes) 10, glucose 10 and $CaCl_2$ 1.2, pH adjusted to 7.4. All experiments were performed at room temperature (22–25°C). In experiments where Ca^{2+}-free medium are indicated, Ca^{2+} was omitted and 1 mmol l^{-1} EGTA was added.

Cell loading and $[Ca^{2+}]_c$ determination

After isolation, the cells were suspended in physiological solution (same composition as before) and loaded with the fluorescent ratiometric Ca^{2+} indicator fura-2 by incubation with 4 μmol l^{-1} fura-2 acetoxymethyl ester at room temperature (23–25°C) for 25–30 min. Once loaded, the cells were washed and resuspended in fresh physiological solution and used within the next 2–4 h. Ca^{2+}-dependent fluorescence signals were monitored in samples of fura-2-loaded cells placed on a thin glass coverslip attached to a Perspex perfusion chamber on the stage of an epifluorescence inverted microscope (Nikon diaphot T200, Kawasaki, Kanagawa, Japan). Perfusion (a flow rate of 1.5 ml min^{-1}) at room temperature was started after a 5 min period to allow spontaneous attachment of the cell to the coverslip. No coating treatment was necessary to immobilize the cells. For quantification of fluorescence, samples were alternatively

excited at 340 and 380 nm using a high-speed monochromator (Polychrome IV) with an integrated light source from a xenon lamp (UXL S/50 MO) (Tills Photonics GmbH, Munich, Germany). Fluorescence emission at 505 nm was detected using a high-speed cooled digital CCD camera (C-4880-81, Hamamatsu Photonics, Marimoto, Shizuoka, Japan) and recorded using dedicated software (Aquacosmos 2.5, Hamamatsu Photonics). Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 ratio and calibrated according to published methods (Grynkiewicz et al., 1985).

Cell viability

Cell viability was assessed using calcein-fluorescence and the Trypan Blue exclusion test. For calcein loading, cells were incubated for 30 min with 5 μmol l^{-1} acetoxymethyl (calcein AM) at 37°C, centrifuged, and the pellet resuspended in fresh buffer. Fluorescence was recorded from 2 ml samples using a fluorescence spectrophotometer (Varian, Ltd., Madrid, Spain). Samples were excited at 494 nm and the resulting fluorescence was measured at 535 nm. After treatment with 1 mmol l^{-1} *t*BHP or agonists, cells were centrifuged and resuspended in fresh buffer. The calcein fluorescence remaining in the cells after treatment with *t*BHP was the same as in controls, at least for the duration of our experiments, suggesting that under our conditions there was no cellular plasma membrane damage. The results obtained with calcein were confirmed using the Trypan Blue exclusion technique. 95% of cells were viable after treatment with *t*BHP similar to that observed in our resting acinar cells suspension. However, when the cells were perfused with 1 mmol l^{-1} *t*BHP for a period longer than 40–45 min, their viability was reduced to 89% and the fura-2 fluorescence suddenly decreased, suggesting that during this period *t*BHP can damage cell permeability and the fluorescence from fura-2 is lost to the extracellular solution.

Statistical analysis

Analyses of statistical significance were performed using Student's *t*-test. Differences were considered significant at $P<0.05$.

Results

In the presence of normal extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$=1.2 mmol l^{-1}), perfusion of pancreatic acinar cells with 1 mmol l^{-1} *t*BHP caused a slow and sustained $[Ca^{2+}]_c$ increase, which reached a stable $[Ca^{2+}]_c$ plateau after 20–25 min of perfusion (Fig. 1A). Fig. 1B shows that the

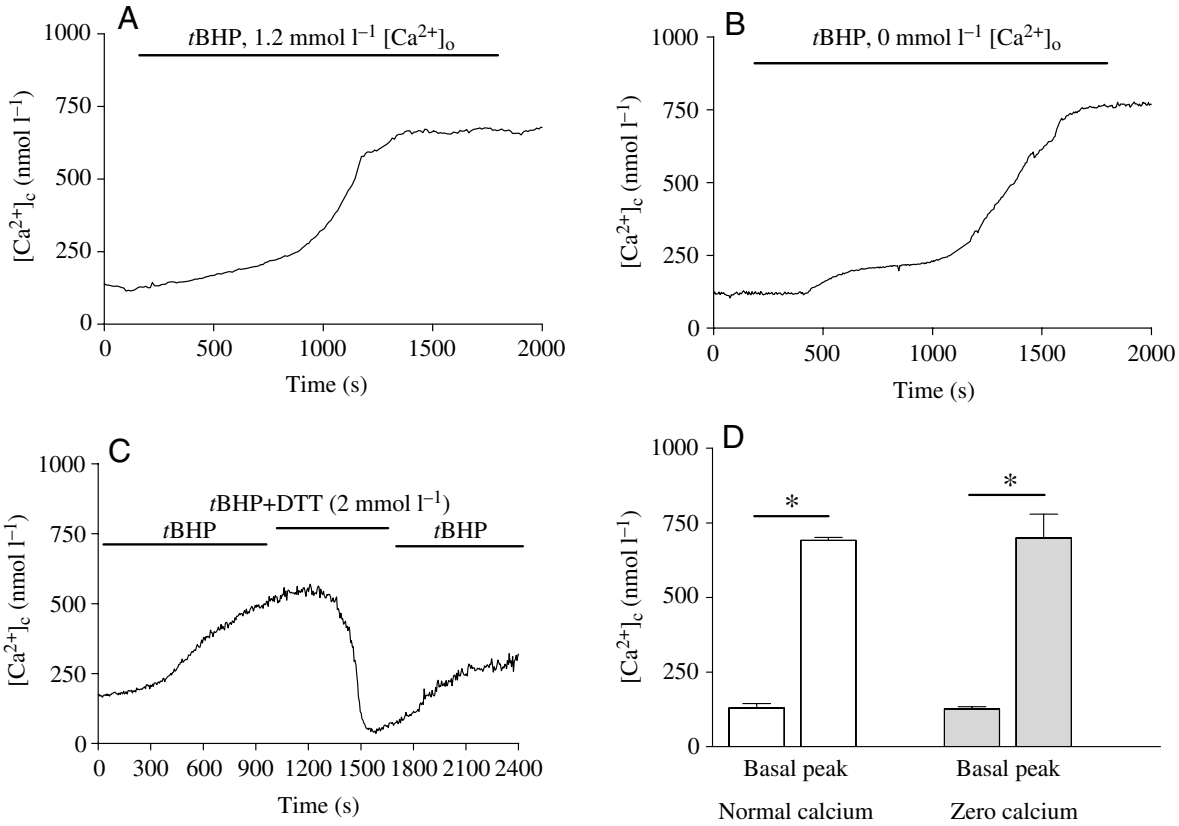

Fig. 1. *t*BHP-evoked $[Ca^{2+}]_c$ increase in isolated rat pancreatic acinar cells. Cells were perfused with 1 mmol l^{-1} *t*BHP in (A) normal-Ca^{2+} or (B) Ca^{2+}-free (containing 1 mmol l^{-1} EGTA) medium. (C) Cells were perfused with 1 mmol l^{-1} *t*BHP followed by 2 mmol l^{-1} DTT in Ca^{2+}-free medium. Traces are representative of 61 and 53 such cells taken from 10 and 8 different experiments, respectively. (D) Histogram represents the mean post-stimulus $[Ca^{2+}]_c$ under different experimental conditions of 8–10 independent experiments. Values are means ± s.e.m. *$P<0.05$.

increase of $[Ca^{2+}]_c$ induced by *t*BHP was also observed in a Ca^{2+}-free medium ($[Ca^{2+}]_o$=0 mmol l^{-1}), reflecting the release of Ca^{2+} from intracellular store(s). The Ca^{2+} released by *t*BHP both in the presence and absence of extracellular Ca^{2+} was statistically similar (691±10 mol l^{-1} *versus* 699±79 mol l^{-1}, Fig. 1D). Fig. 1B also shows that *t*BHP caused a biphasic increase in $[Ca^{2+}]_c$, consisting of a slow initial rise observed within the first minutes and a second sustained rise later during the course of experiments. A significant proportion of the cells studied exhibited this biphasic transient increase in $[Ca^{2+}]_c$ (36 of 53 examined cells, 67.92%). The Ca^{2+} release induced by 1 mmol l^{-1} *t*BHP was reversed by an addition of 2 mmol l^{-1} of the reducing agent dithiothreitol (DTT) (Fig. 1C). This inhibition was reversible; removal of DTT allowed $[Ca^{2+}]_c$ to return to the transient increase evoked by *t*BHP.

Pretreatment of cells with *t*BHP abolished the typical Ca^{2+} response both to the Ca^{2+}-mobilising agonist CCK-8 (1 nmol l^{-1}) (Fig. 2A) and to TPS (1 μmol l^{-1}), a specific inhibitor of SERCA (Fig. 2B), in the absence of extracellular Ca^{2+}. However, when the agonist-releasable Ca^{2+} pools had previously been depleted by a maximal concentration (1 nmol l^{-1}) of CCK-8 (Fig. 3A) or 1 μmol l^{-1} thapsigargin (TPS) (Fig. 3B) in a Ca^{2+}-free solution, 1 mmol l^{-1} *t*BHP was still able to induce Ca^{2+} release in 66 of 74 examined cells (89.18%) and in 41 of 58 examined cells (70.69%) from 12 and 10 experiments, respectively (Fig. 3), suggesting that *t*BHP is also able to release Ca^{2+} from an agonist-insensitive

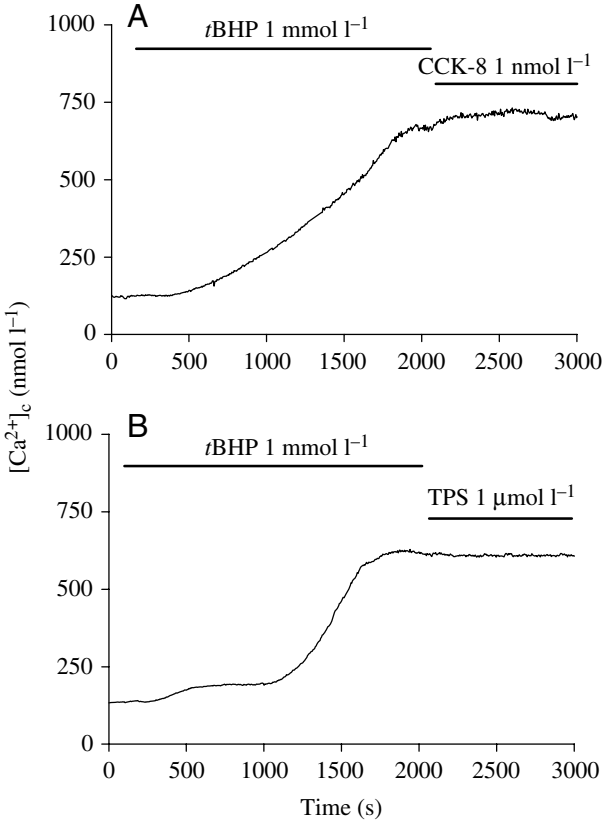

Fig. 2. Mobilisation of Ca^{2+} in response to agonists in isolated rat pancreatic acinar cells pretreated with *t*BHP. Cells were initially perfused with 1 mmol l^{-1} *t*BHP followed by 1 nmol l^{-1} CCK-8 (A) or 1 μmol l^{-1} thapsigargin (TPS) (B) in Ca^{2+}-free medium. Traces are representative of 32 and 26 such cells taken from 12 and 10 different experiments, respectively.

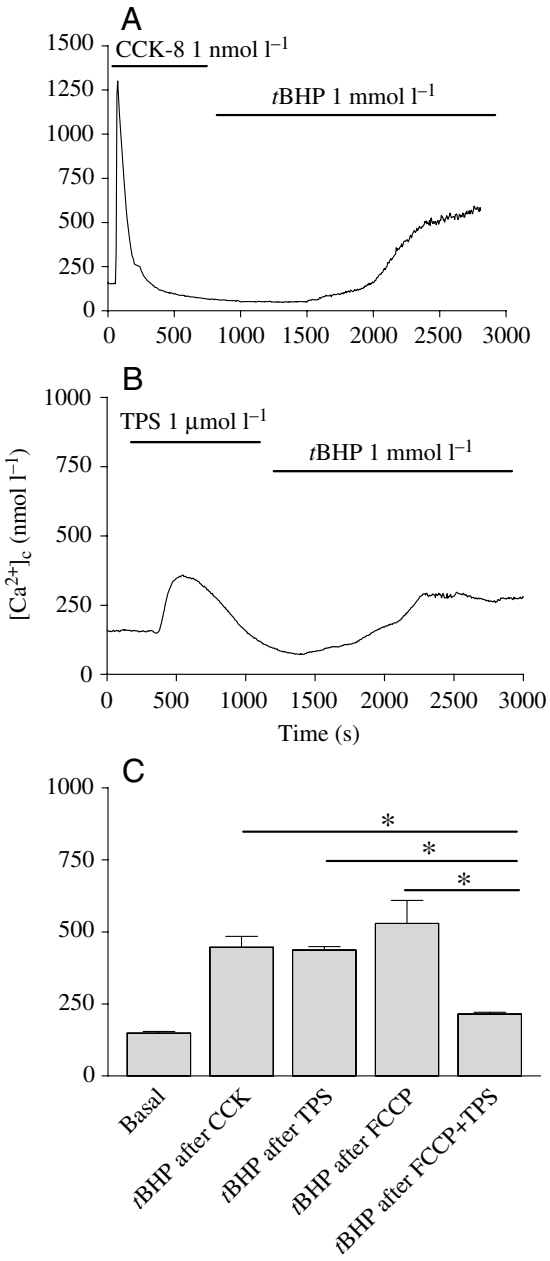

Fig. 3. Effect of agonist-induced depletion of cytosolic Ca^{2+} stores on *t*BHP-evoked Ca^{2+} increase in pancreatic acinar cells. Cells were perfused with either 1 nmol l^{-1} CCK-8 (A) or 1 μmol l^{-1} thapsigargin (TPS) (B) in Ca^{2+}-free medium, followed by 1 mmol l^{-1} *t*BHP. Traces are representative of 74 and 58 such cells taken from 12 and 10 different experiments, respectively. (C) Histogram of the mean post-stimulus $[Ca^{2+}]_c$ under different experimental conditions in 5–12 independent experiments. Values are means ± s.e.m. **P*<0.05.

store. The amount of Ca^{2+} released by *t*BHP after treatment with CCK or TPS is shown in Fig. 3C.

In order to investigate the nature of the *t*BHP-releasable agonist-insensitive Ca^{2+} store we used FCCP, a mitochondrial uncoupler that collapses the mitochondrial membrane potential that drives Ca^{2+} uptake (Buckler and Vaughan-Jones, 1998). As shown in Fig. 4A, pretreatment of the acinar cells with 0.5 μmol l^{-1} FCCP in a Ca^{2+}-free medium resulted in a sustained increase in $[Ca^{2+}]_c$ due to release of Ca^{2+} from mitochondrial stores. Subsequent addition of 1 mmol l^{-1} *t*BHP, the acinar cell suspension was still able to release Ca^{2+}, presumably from agonist-sensitive stores (21 of 27 examined cells, 77.77%, from 7 experiments). Pretreatment with *t*BHP abolished Ca^{2+} release from mitochondria evoked by subsequent addition of FCCP (Fig. 4B) (in all 18 recorded cells, from 6 experiments), suggesting that mitochondrial stores are depleted by pretreatment with *t*BHP. By contrast, simultaneous addition of 1 μmol l^{-1} TPS and 0.5 μmol l^{-1} FCCP (which deplete non-mitochondrial intracellular Ca^{2+} stores, e.g. endoplasmic reticulum and mitochondria, respectively) clearly abolished the *t*BHP-induced Ca^{2+} increase in all 22 cells examined from 5 experiments (Figs 5, 3C). Taken together, these findings indicate that *t*BHP releases Ca^{2+} from both mitochondrial and non-mitochondrial Ca^{2+} pools.

Since it had been previously shown that oxidising reagents are able to sensitize IP_3-induced Ca^{2+} release (Thorn et al., 1992; Wu et al., 1996), we also wanted to evaluate whether *t*BHP can release Ca^{2+} from agonist-mobilisable Ca^{2+} stores by sensitising the IP_3-induced Ca^{2+} release. To test this possibility we employed 2-aminoethoxydiphenylborane (2-APB), a blocker of IP_3-mediated Ca^{2+} release that does not interact with the IP_3-binding site (Soulsby and Wojcikiewicz, 2002), and which was able to block the Ca^{2+} signal evoked by the Ca^{2+}-mobilising agonist CCK-8 (data not shown). As shown in Fig. 6A, application of 30 μmol l^{-1} 2-APB to acinar cells, where the mitochondrial Ca^{2+} pool had been previously depleted by 0.5 μmol l^{-1} FCCP to avoid interference with mitochondrial Ca^{2+} release, was unable to suppress the increase in $[Ca^{2+}]_c$ induced by 1 mmol l^{-1} *t*BHP in 26 of 37 cells examined (70.27%) from three experiments.

The effects of ryanodine were also examined in order to investigate the putative implication of ryanodine receptors on *t*BHP-evoked Ca^{2+} mobilisation. In both excitable and non-excitable cells, ryanodine at relatively low concentrations (10 nmol l^{-1}–10 μmol l^{-1}) is reported to cause activation of the Ca^{2+} release channel, whereas at higher concentrations (>10 μmol l^{-1}) ryanodine blocks channel activation (Verkhratsky and Shmigol, 1996). In our experimental conditions, pretreatment of pancreatic acinar cells, whose mitochondrial Ca^{2+} stores had been depleted using FCCP (0.5 μmol l^{-1}), with 50 μmol l^{-1} ryanodine (which blocked caffeine-evoked Ca^{2+} release, data not shown) abolished *t*BHP-evoked Ca^{2+} mobilisation in 35 of 46

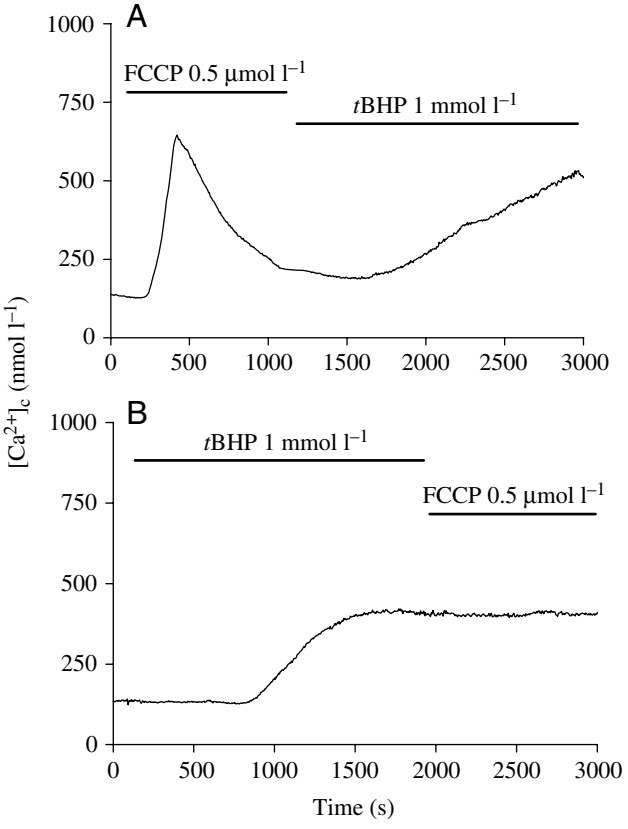

Fig. 4. Effect of depletion of Ca^{2+} mitochondrial pools on *t*BHP-evoked Ca^{2+} release in pancreatic acinar cells. (A) Cells were perfused with 0.5 μmol l^{-1} FCCP, followed by perfusion with 1 mmol l^{-1} *t*BHP, in Ca^{2+}-free medium. (B) Cells were perfused with 1 mmol l^{-1} *t*BHP, followed by perfusion with 0.5 μmol l^{-1} FCCP, in Ca^{2+}-free medium. Traces are representative of 27 and 18 such cells taken from 7 and 6 different experiments, respectively.

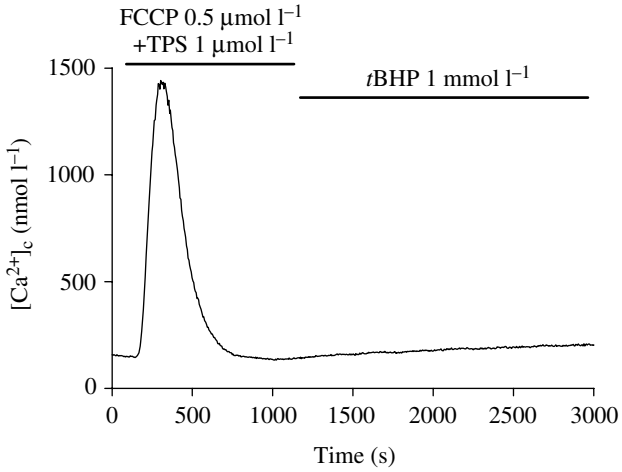

Fig. 5. Effect of depletion of mitochondrial and non-mitochondrial intracellular Ca^{2+} stores on *t*BHP-evoked Ca^{2+} release. Pancreatic acinar cells were perfused with 0.5 μmol l^{-1} FCCP plus 1 μmol l^{-1} thapsigargin (TPS), followed by 1 mmol l^{-1} *t*BHP in a Ca^{2+}-free medium. Trace is representative of 22 such cells taken from 5 experiments.

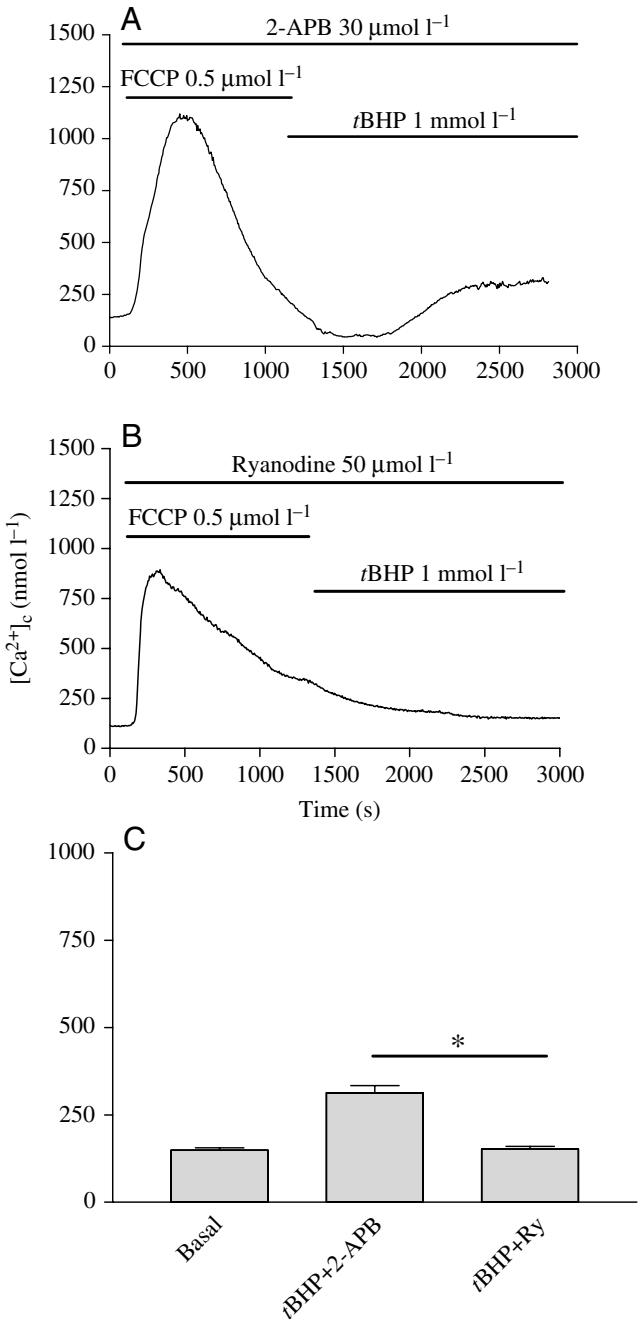

Fig. 6. Changes in the cytosolic Ca^{2+} mobilisation of pancreatic acinar cells in response to perfusion with FCCP (0.5 μmol l^{-1}), followed by *t*BHP (1 mmol l^{-1}) in the presence of (A) 2-aminoethoxydiphenylborane (2-APB, 30 μmol l^{-1}) or (B) ryanodine (50 μmol l^{-1}). All experiments were performed in a Ca^{2+}-free solution (1 mmol l^{-1} EGTA was added). Traces are representative of 37 and 46 cells taken from 2 and 3 different experiments, respectively. (C) Histogram of the mean post-stimulus $[Ca^{2+}]_c$ under different experimental conditions of 2–3 independent experiments. Values are means ± s.e.m. *P<0.05.

examined cells (76.08%) from 3 experiments (Fig. 6B,C), suggesting that *t*BHP releases Ca^{2+} from non-mitochondrial Ca^{2+} pools through ryanodine channels.

Discussion

In this study we demonstrate that the membrane-permeant oxidant *t*BHP induces a $[Ca^{2+}]_c$ increase in pancreatic acinar cells by Ca^{2+} release from intracellular stores, since this effect was observed in a Ca^{2+}-free medium. By contrast, pretreatment of acinar cells with *t*BHP followed by the addition of either CCK-8 or TPS resulted in an abolition of the agonist-evoked rise in $[Ca^{2+}]_c$, whereas *t*BHP failed to increase $[Ca^{2+}]_c$ in cells where non-mitochondrial and mitochondrial intracellular Ca^{2+} stores had previously been depleted by application of TPS plus FCCP, in a Ca^{2+}-free solution. Our results are consistent with those previously described by us in isolated rat pancreatic acinar cells and platelets treated with H_2O_2 (Pariente et al., 2001; Redondo et al., 2004). In addition, our results seem to indicate that *t*BHP acts by mobilising Ca^{2+} from non-mitochondrial pools *via* an IP_3-receptor independent mechanism that involves ryanodine channels.

On the basis of its ability to increase $[Ca^{2+}]_c$, ROS have been considered to be pathogenic factors in different tissues, including the pancreas (Weber et al., 1998). An increase in $[Ca^{2+}]_c$ due to disturbance of Ca^{2+} homeostasis by ROS can cause morphological and functional alterations to the cells, and therefore, have been clearly established as contributing to disease and cell death (Jacobson and Duchen, 2002). Impairment of Ca^{2+} homeostasis and intrapancreatic activation of digestive enzymes have been proposed as critical events in the development of pancreatitis (Saluja et al., 1999). In addition, high levels of ROS have been implicated as important mediators in the pathogenesis of acute pancreatitis. Thus, it was of interest to analyse Ca^{2+} homeostasis in cells exposed to oxidative conditions.

In pancreatic acinar cells, the stimulatory effect of *t*BHP on resting $[Ca^{2+}]_c$ and its inhibitory effect on agonist-induced Ca^{2+} mobilisation could be due to a direct effect on the Ca^{2+} release process and not a consequence of the opposing action in the Ca^{2+} pathway. Previous studies in different cell types, such as hepatocytes (Miyoshi et al., 1996; Byrne et al., 1999), erythrocytes (Lang et al., 2003), platelets (Elferink, 1999; Redondo et al., 2004), and endothelial cells (Elliot et al., 1989; Jornot et al., 1999), have reported that hydroperoxides and other sulphydryl reagents can induce Ca^{2+} mobilisation.

Other authors have shown that the sulphydryl group oxidising agents thimerosal (Thorn et al., 1992), vanadate (Pariente et al., 1999) and phenylarsine oxide (Lajas et al., 1999) are able to mobilise Ca^{2+} from intracellular stores in pancreatic acinar cells and that this effect is reversed in the presence of the thiol-reducing agent dithiothreitol. Similar results were obtained in thymus cells (Calviello et al., 1993) and hepatocytes (Nicotera et al., 1988) using *t*BHP as oxidising agent. Additionally, the depletion of intracellular stores by *t*BHP has been observed in other cell types, such as hepatocytes (Masaki et al., 1989), PC12 pheochromocytoma cells (Lu et al., 2002), alveolar macrophages (Hoyal et al., 1996), skeletal muscle (Silva et al., 1997), myeloid leukaemia U937 cells (Clementi et al., 1998) or neuronal cell line SH-SY5Y (Amoroso et al., 1999). However, evidence exists that

*t*BHP increases $[Ca^{2+}]_c$ exclusively *via* Ca^{2+} influx from the extracellular site (Kim et al., 1998). This Ca^{2+} entry can occur through voltage-dependent Ca^{2+} channels (Wahl et al., 1998). Other authors indicate that the *t*BHP-induced effect might be mediated both by Ca^{2+} influx from the extracellular medium and by intracellular store depletion (Bernardes et al., 1986; Teplova et al., 1998).

Our results show that *t*BHP releases Ca^{2+} from intracellular stores, suggesting that the failure of CCK-8 and TPS to induce Ca^{2+} mobilisation after *t*BHP is related to a partial or complete depletion of the stores by this agent. The *t*BHP-sensitive Ca^{2+} pools include those released by TPS (e.g. endoplasmic reticulum) and FCCP (e.g. mitochondria). This is shown by the failure of *t*BHP to increase $[Ca^{2+}]_c$ after treatment with TPS plus FCCP in a Ca^{2+}-free medium. Thus, when the non-mitochondrial agonist-releasable Ca^{2+} pools are previously depleted by CCK-8 or TPS, *t*BHP is able to induce Ca^{2+} release from mitochondria in a Ca^{2+}-free medium, whereas if the mitochondrial Ca^{2+} is released by treatment with FCCP, *t*BHP releases the Ca^{2+} from the TPS-sensitive pool. In this context, it is important to note that the existence of two major types of intracellular Ca^{2+} stores has been suggested: (i) the endoplasmic reticulum, which functions as a high-affinity, low-capacity Ca^{2+} pool, and (ii) mitochondria, which are low-affinity, high-capacity Ca^{2+} pools (Carafoli, 1987).

The existence of two intracellular Ca^{2+} pools could also explain the biphasic transient increase in $[Ca^{2+}]_c$ induced by *t*BHP in the majority of our cells by sequential depletion of both pools. One of the two rises in $[Ca^{2+}]_c$ could be due to mobilisation of Ca^{2+} from endoplasmic reticulum or mitochondria. The initial phase might be due to release of non-mitochondrial Ca^{2+}, and the second to the release of mitochondrial Ca^{2+}. It is also worth noting that once the non-mitochondrial pool is depleted, *t*BHP causes a slow Ca^{2+} release (corresponding to the mitochondrial store) (Fig. 3A,B), whereas when the mitochondrial store is already depleted the *t*BHP effect is much faster (Fig. 4A), as would be expected if the Ca^{2+} was released from the non-mitochondrial pool.

Our findings, in which *t*BHP releases Ca^{2+} from intracellular stores, are consistent with previous reports where the $[Ca^{2+}]_c$ increase evoked by *t*BHP is accomplished by an inhibition of the PMCA (Hoyal et al., 1996) and/or by sensitisation of the sarcoplasmic reticulum Ca^{2+} release channels (Lang et al., 2003; Redondo et al., 2004). In fact, it has been reported that both *t*BHP metabolism to radical species and/or accumulation of oxidised glutathione can damage Ca^{2+}-ATPase functions in the plasma membrane and the endoplasmic reticulum (Viner et al., 1997). Furthermore, the opening of these channels has been shown to be modulated by numerous factors, including phosphorylation, adenine nucleotides, thiol reactive compounds and pH (Bootman et al., 2001). Redox modulation of channel activity has been previously reported in various channels (DiChiara and Reinhart, 1997). The endoplasmic reticulum, a key organelle in cytosolic Ca^{2+} signal generation, expresses two separate and related families of Ca^{2+}-release channels, inositol 1,4,5-triphosphate (IP_3R) and ryanodine (RyR) receptors (Ashby and Tepikin, 2002; Bootman et al., 2002), and it is largely responsible for mediating Ca^{2+} release from intracellular stores. One type of intracellular Ca^{2+} pool is sensitised by IP_3, which activates IP_3-induced Ca^{2+} release (IICR). Another is sensitised by ryanodine, leading to a Ca^{2+}-induced Ca^{2+} release (CICR) process (Petersen and Wakui, 1990).

It has been demonstrated in several cell types that the presence of different oxidising reagents 'sensitise' RyR and IP_3R, through blocker or stimulative mechanisms (Suko et al., 2000; Schultheiss et al., 2005). In pancreatic β-cells, thiol oxidation by the reactive disulphide 2,2′-dithiodipyridine causes a release of Ca^{2+} from intracellular stores by mechanisms that do not involve activation of RyR, but occur from the IP_3-sensitive intracellular Ca^{2+} pools (Islam et al., 1997). Additionally, in pancreatic acinar cells it has been shown that free radicals generated by xanthine oxidase-catalyzed oxidation of hypoxanthine are able to mobilise Ca^{2+} from ryanodine-sensitive intracellular stores (Klonoswki-Stumpe et al., 1997). Our results using 2-APB (at a concentration of 30 μmol l^{-1}, known to block the IP_3R) indicate that *t*BHP releases Ca^{2+} from a non-mitochondrial Ca^{2+} pool by an IP_3R-independent mechanism. Similar results were obtained previously by us (Pariente et al., 2001) and by others (Hoyal et al., 1996; Clementi et al., 1998). This conclusion is supported by our results showing that ryanodine (at a concentration of 50 μmol l^{-1}, which blocks ryanodine receptors), abolishes *t*BHP-induced Ca^{2+}-release from non-mitochondrial Ca^{2+} pools, thus suggesting that *t*BHP sensitises ryanodine receptors, at least in pancreatic acinar cells. In fact, it has been reported that other oxidising agents, like H_2O_2, release Ca^{2+} from intracellular stores by activation of the ryanodine receptor (Favero et al., 1995; Oba et al., 1998) and that sulphydryl groups (susceptible to oxidation) have a critical role in the ryanodine-sensitive Ca^{2+} channel (Oba et al., 1998). In addition, ryanodine shows '*in vitro*' sensitisation in the presence of the sulphydryl group oxidising agent thimerosal (Abramson et al., 1995; Wu et al., 1996).

In summary, our findings show that treatment of pancreatic acinar cells with *t*BHP results in the release of Ca^{2+} from mitochondrial and non-mitochondrial intracellular stores, *via* ryanodine-sensitive channels. From a physiological point of view, these results help us to understand the complex mechanism of intracellular Ca^{2+} homeostasis in pancreatic acinar cells.

List of abbreviations

$[Ca^{2+}]_c$	cytosolic free Ca^{2+} concentration
$[Ca^{2+}]_o$	extracellular Ca^{2+} concentration
2-APB	2-aminoethoxydiphenyl borane
CCK-8	cholecystokinin octapeptide
CICR	Ca^{2+}-induced Ca^{2+} release
DTT	dithiothreitol
ER/SR	endoplasmic reticulum/sarcoplasmic reticulum
FCCP	carbonyl cyanide p-trifluoromethoxy-phenylhydrazone

H_2O_2	hydrogen peroxide
IICR	IP_3-induced Ca^{2+} release
IP_3	inositol-1,4,5-triphosphate
IP_3R	inositol-1,4,5-triphosphate receptor
PMCA	plasma membrane Ca^{2+}-pump ATPase
ROS	reactive oxygen species
RyR	ryanodine receptor
SERCA	sarcoendoplasmic reticulum Ca^{2+} ATPase
*t*BHP	*tert*-butylhydroperoxide
TPS	thapsigargin

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References

Abramson, J. J., Zable, A. C., Favero, T. G. and Salama, G. (1995). Thimerosal interacts with the Ca^{2+} release channel ryanodine receptor from skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **270**, 29644-29647.

Amoroso, S., Gioielli, A., Cataldi, M., Di Renzo, G. and Annunziato, L. (1999). In the neuronal cell line SH-SY5Y, oxidative stress-induced free radical overproduction causes cell death without any participation of intracellular Ca^{2+} increase. *Biochim. Biophys. Acta* **1452**, 151-160.

Ashby, M. C. and Tepikin, A. V. (2002). Polarized calcium and calmodulin signaling in secretory epithelia. *Physiol. Rev.* **82**, 701-734.

Bernardes, C. F., Pereira da Silva, L. and Vercesi, A. E. (1986). *t*-Butylhydroperoxide-induced Ca^{2+} efflux from liver mitochondria in the presence of physiological concentrations of Mg^{2+} and ATP. *Biochim. Biophys. Acta* **850**, 41-48.

Berridge, M. J., Lipp, P. and Bootman, M. D. (2000). The versatility and universatility of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **1**, 11-21.

Bootman, M. D., Collins, T. J., Peppiatt, C. M., Prothero, L. S., MacKenzie, L., De Smet, P., Travers, M., Tovey, S. C., Seo, J. T., Berridge, M. J. et al. (2001). Calcium signalling – an overview. *Semin. Cell Dev. Biol.* **12**, 3-10.

Bootman, M. D., Berridge, M. J. and Roderick, H. L. (2002). Calcium signalling: more messengers, more channels, more complexity. *Curr. Biol.* **12**, 563-565.

Buckler, K. J. and Vaughan-Jones, R. D. (1998). Effects of mitochondrial uncouplers on intracellular calcium, pH and membrane potential in rat carotid body type I cells. *J. Physiol.* **513**, 819-833.

Byrne, A. M., Lemasters, J. J. and Nieminen, A. L. (1999). Contribution of increased mitochondrial free Ca^{2+} to the mitochondrial permeability transition induced by tert-butylhydroperoxide in rat hepatocytes. *Hepatology* **29**, 1523-1531.

Calviello, G., Ricci, P. and Bartola, G. M. (1993). *tert*-butyl hydroperoxide induced $[Ca^{2+}]_i$ increase in thymus and thymoma cells. *Biochem. Biophys. Res. Commun.* **197**, 859-868.

Camello, P., Gardner, J., Petersen, O. H. and Tepikin, A. V. (1996). Calcium dependence of calcium extrusion and calcium uptake in mouse pancreatic acinar cells. *J. Physiol.* **490**, 585-593.

Carafoli, E. (1987). Intracellular calcium homeostasis. *Annu. Rev. Biochem.* **56**, 395-433.

Carafoli, E., Santella, L., Branca, D. and Brini, M. (2001). Generation, control, and processing of cellular calcium signals. *Crit. Rev. Biochem. Mol. Biol.* **36**, 107-260.

Castilho, R. F., Carvalho-Alves, P. C., Vercesi, A. E. and Ferreir, S. T. (1996). Oxidative damage to sarcoplasmic reticulum Ca^{2+}-pump induced by Fe^{2+}/H_2O_2/ascorbate is not mediated by lipid peroxidation or thiol oxidation and leads to protein fragmentation. *Mol. Cell. Biochem.* **159**, 105-114.

Clementi, E., Guidarelli, A. and Canton, O. (1998). The inositol 1,4,5-trisphosphate-generating agonist ATP enhances DNA cleavage induced by *tert*-butylhydroperoxide. *Exp. Cell Res.* **239**, 175-178.

DiChiara, T. J. and Reinhart, P. H. (1997). Redox modulation of hslo Ca^{2+}-activated K^+ channels. *J. Neurosci.* **17**, 4942-4955.

Distelhorst, C. W. and Dubyak, G. (1998). Role of calcium in glucocorticosteroid-induced apoptosis of thymocytes and lymphoma cells: resurrection of old theories by new findings. *Blood* **91**, 731-734.

Doan, T. N., Gentry, D. L., Taylor, A. A. and Elliott, S. J. (1994). Hydrogen peroxide activates agonist-sensitive Ca^{2+}-flux pathways in canine venous endothelial cells. *Biochem. J.* **297**, 209-215.

Duchen, M. R. (2000). Mitochondria and calcium: from cell signalling to cell death. *J. Physiol.* **529**, 57-68.

Elferink, J. G. (1999). Thimerosal: a versatile sulfhydryl reagent, calcium mobilizer, and cell function-modulating agent. *Gen. Pharmacol.* **33**, 1-6.

Elliott, S. J., Eskin, S. G. and Scchilling, W. P. (1989). Effect of t-Butyl-hydroperoxide on Bradykinin-stimulated changes in cytosolic calcium in vascular endothelial cells. *J. Biol. Chem.* **264**, 3806-3810.

Favero, T. G., Zable, A. C. and Abramson, J. J. (1995). Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* **270**, 25557-25563.

Gerasimenko, J. V., Gerasimenko, O. V., Palejwala, A., Tepikin, A. V., Petersen, O. H. and Watson, A. J. M. (2002). Menadione-induced apoptosis: roles of cytosolic Ca^{2+} elevations and the mitochondrial permeability transition pore. *J. Cell Sci.* **115**, 485-497.

González, A. and Salido, G. M. (2001). Participation of mitochondria in calcium signalling in the exocrine pancreas. *J. Physiol. Biochem.* **57**, 331-339.

González, A., Granados, M. P., Salido, G. M. and Pariente, J. A. (2005). H_2O_2-induced changes in mitochondrial activity in isolated mouse pancreatic acinar cells. *Mol. Cell. Biochem.* **269**, 165-173.

Gorbunov, N. V., Tyurina, V. Y., Salama, G., Day, B. W., Claycamp, H. G., Argüiros, G., Elsayed, N. M. and Kagan, V. E. (1998). Nitric oxide protects cardiomyocytes against *tert*-butyl hydroperoxide-induced formation of alkoxyl and peroxyl radicals and peroxidation of phosphatidylserine. *Biochem. Biophys. Res. Commun.* **244**, 647-651.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**, 3440-3450.

Hoyal, C. R., Thomas, A. P. and Forman, H. J. (1996). Hydroperoxide-induced increases in intracellular calcium due to annexin VI translocation and inactivation of plasma membrane Ca^{2+}-ATPase. *J. Biol. Chem.* **271**, 29205-29210.

Islam, M. S., Kindmark, H., Larsson, O. and Berggren, P. O. (1997). Thiol oxidation by 2,2′-dithiodipyridine causes a reversible increase in cytoplasmic free Ca^{2+} concentration in pancreatic beta-cells. Role for inositol 1,4,5-trisphosphate-sensitive Ca^{2+} stores. *Biochem. J.* **321**, 347-354.

Jacobson, J. and Duchen, M. R. (2002). Mitochondrial oxidative stress and cell death in astrocytes-requirement for Ca^{2+} and sustained opening of the permeability transition pore. *J. Cell Sci.* **115**, 1175-1188.

Jones, D. P., Thor, H., Smith, M. T., Jewell, S. A. and Orrenius, S. (1983). Inhibition of ATP-dependent microsomal Ca^{2+} sequestration during oxidative stress and its prevention by glutathione. *J. Biol. Chem.* **258**, 6390-6393.

Jornot, L., Maechler, P., Wollheim, C. B. and Junod, A. F. (1999). Reactive oxygen metabolites increase mitochondrial calcium in endothelial cells: implication of the Ca^{2+}/Na^+ exchanger. *J. Cell Sci.* **112**, 1013-1022.

Kim, J. A., Kang, Y. S., Kim, Y. O., Lee, S. H. and Lee, Y. S. (1998). Role of Ca^{2+} influx in the tert-butyl hydroperoxide-induced apoptosis of HepG2 human hepatoblastoma cells. *Exp. Mol. Med.* **30**, 137-144.

Klonowski-Stumpe, H., Schreiber, R., Grolik, M., Schulz, H. U., Haussinger, D. and Niederau, C. (1997). Effect of oxidative stress on cellular functions and cytosolic free calcium of rat pancreatic acinar cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **272**, G1489-G1498.

Lajas, A. I., Pozo, M. J., Camello, P. J., Salido, G. M. and Pariente, J. A. (1999). Phenylarsine oxide evokes intracellular calcium increases and amylase secretion in isolated rat pancreatic acinar cells. *Cell. Signal.* **11**, 727-734.

Lang, K. S., Duranton, C., Poehlmann, H., Myssina, S., Bauer, C., Lang, F., Wieder, T. and Huber, S. M. (2003). Cation channels trigger apoptotic death of erythrocytes. *Cell Death Differ.* **10**, 249-256.

Liu, H., Miller, E., van de Water, B. and Stevens, J. L. (1998). Endoplasmic reticulum stress proteins block oxidant-induced Ca^{2+} increases and cell death. *J. Biol. Chem.* **273**, 12858-12862.

Lu, C. H., Su, W., Lo, Y. K., Chen, W. C., Chang, W. N., Wang, J. L., Tsai, Y. C., Lee, P. Y. and Jan, C. R. (2002). Effect of *t*-butyl

hydroperoxide on Ca^{2+} movement in PC12 pheochromocytoma cells. *Chin. J. Physiol.* **45**, 51-56.

Martín, C., Martínez, R., Navarro, R., Ruiz-Sanz, J. I., Lacort, M. and Ruiz-Larrea, M. B. (2001). *tert*-Butyl hydroperoxide-induced lipid signaling in hepatocytes: involvement of glutathione and free radicals. *Biochem. Pharmacol.* **62**, 705-712.

Martínez, M., Lajas, A., Yago, M. D., Redondo, P., Granados, M., González, A., Rosado, J., Martínez-Victoria, E., Mañas, M. and Pariente, J. (2004). Dietary virgin olive oil enhances secretagogue-evoked calcium signaling in rat pancreatic acinar cells. *Nutrition* **20**, 536-541.

Masaki, N., Kyle, M. E. and Farber, J. L. (1989). *tert*-butyl hydroperoxide kills cultured hepatocytes by peroxidizing membrane lipids. *Arch. Biochem. Biophys.* **269**, 390-399.

Miyoshi, H., Umeshita, K., Sakon, M., Imajoh-Ohmi, S., Fujitani, K., Gotoh, M., Oiki, E., Kambayashi, J. and Monden, M. (1996). Calpain activation in plasma membrane bleb formation during tert-butyl hydroperoxide-induced rat hepatocyte injury. *Gastroenterology* **110**, 1897-1904.

Moreau, V. H., Castilho, R. F., Ferreira, S. T. and Carvalho-Alves, P. C. (1998). Oxidative damage to sarcoplasmic reticulum Ca^{2+}-ATPase AT submicromolar iron concentrations: evidence for metal-catalyzed oxidation. *Free Radic. Biol. Med.* **25**, 554-560.

Nicotera, P., McConkey, D., Svensson, S. A., Bellomo, G. and Orrenius, S. (1988). Correlation between cytosolic Ca^{2+} concentration and cytotoxicity in hepatocytes exposed to oxidative stress. *Toxicology* **52**, 55-63.

Oba, T., Ishikawa, T. and Yamaguchi, M. (1998). Sulfhydryls associated with H_2O_2-induced channel activation are on luminal side of ryanodine receptors. *Am. J. Physiol.* **274**, C914-C921.

Pariente, J. A., Lajas, A. I., Pozo, M. J., Camello, P. J. and Salido, G. M. (1999). Oxidizing effects of vanadate on calcium mobilization and amylase release in rat pancreatic acinar cells. *Biochem. Pharmacol.* **58**, 77-84.

Pariente, J. A., Camello, C., Camello, P. J. and Salido, G. M. (2001). Release of calcium from mitochondrial and non-mitochondrial intracellular stores in mouse pancreatic acinar cells by hydrogen peroxide. *J. Membr. Biol.* **179**, 27-35.

Petersen, O. H. and Wakui, M. (1990). Oscillating intracellular Ca^{2+} signals evoked by activation of receptors linked to inositol lipid hydrolysis: mechanism of generation. *J. Membr. Biol.* **118**, 93-105.

Putney, J. W., Jr (1988). The role of phosphoinositide metabolism in signal transduction in secretory cells. *J. Exp. Biol.* **139**, 135-150.

Redondo, P. C., Salido, G. M., Rosado, J. A. and Pariente, J. A. (2004). Effect of hydrogen peroxide on Ca^{2+} mobilisation in human platelets through sulphydryl oxidation dependent and independent mechanisms. *Biochem. Pharmacol.* **67**, 491-502.

Rohn, T. T., Hinds, T. R. and Vincenzi, F. F. (1993). Inhibition of the Ca^{2+} pump of intact red blood cells by *t*-butyl hydroperoxide: importance of glutathione peroxidase. *Biochim. Biophys. Acta* **1153**, 67-76.

Rooney, T. A., Renard, D. C., Sass, E. J. and Thomas, A. P. (1991). Oscillatory cytosolic calcium waves independent of stimulated inositol 1,4,5-trisphosphate formation in hepatocytes. *J. Biol. Chem.* **266**, 12272-12282.

Rosado, J. A., Redondo, P. C., Salido, G. M., Gómez-Arteta, E., Sage, S. O. and Pariente, J. A. (2004). Hydrogen peroxide generation induces pp60src activation in human platelets: evidence for the involvement of this pathway in store-mediated calcium entry. *J. Biol. Chem.* **279**, 1665-1675.

Rush, G. F., Gorski, J. R., Ripple, M. G., Sowinski, J., Bugelski, P. and Hewitt, W. R. (1985). Organic hydroperoxide-induced lipid peroxidation and cell death in isolated hepatocytes. *Toxicol. Appl. Pharmacol.* **78**, 473-483.

Saluja, A. K., Bhagat, L., Lee, H. S., Bhatia, M., Frossard, J. L. and Steer, M. (1999). Secretagogue-induced digestive enzyme activation and cell injury in rat pancreatic acini. *Am. J. Physiol.* **276**, G835-G842.

Schultheiss, G., Lan Kocks, S. and Diener, M. (2005). Stimulation of colonic anion secretion by monochloramine: action sites. *Pflugers Arch.* **449**, 553-563.

Silva, J. P., Winterhalter, K. H. and Richter, C. (1997). *t*-Butylhydroperoxide and gliotoxin stimulate Ca^{2+} release from rat skeletal muscle mitochondria. *Redox Rep.* **3**, 331-341.

Soulsby, M. D. and Wojcikiewicz, R. J. (2002). 2-Aminoethoxydiphenyl borate inhibits inositol 1,4,5-trisphosphate receptor function, ubiquitination and downregulation, but acts with variable characteristics in different cell types. *Cell Calcium* **32**, 175-181.

Suko, J., Hellmann, G. and Drobny, H. (2000). Modulation of the calmodulin-induced inhibition of sarcoplasmic reticulum calcium release channel (ryanodine receptor) by sulfhydryl oxidation in single channel current recordings and [(3)H]ryanodine binding. *J. Membr. Biol.* **174**, 105-120.

Sweiry, J. H., Shibuya, I., Asada, N., Niwa, K., Doolabh, K., Habara, Y., Kanno, T. and Mann, G. E. (1999). Acute oxidative stress modulates secretion and repetitive Ca^{2+} spiking in rat exocrine pancreas. *Biochim. Biophys. Acta* **1454**, 19-30.

Tepikin, A. V., Voronina, S. G., Gallacher, D. V. and Petersen, O. H. (1992). Pulsatile Ca^{2+} extrusion from single pancreatic acinar cells during receptor-activated cytosolic Ca^{2+} spiking. *J. Biol. Chem.* **267**, 14073-14076.

Teplova, V. V., Kudrjavtsev, A. A., Odinokova, I. V., Evtodienko, Y. V. and Saris, N. E. (1998). Effect of prooxidants on mitochondrial permeability transition and cell death in Ehrlich ascites tumour cells. *Biochem. Mol. Biol. Int.* **45**, 501-510.

Thorn, P., Brady, P., Llopis, J., Gallacher, D. V. and Petersen, O. H. (1992). Cytosolic Ca^{2+} spikes evoked by the thiol reagent thimerosal in both intact and internally perfused single pancreatic acinar cells. *Pflugers Arch.* **422**, 173-178.

Verkhratsky, A. and Shmigol, A. (1996). Calcium-induced calcium release in neurones. *Cell Calcium* **19**, 1-14.

Viner, R. I., Ferrington, D. A., Aced, G. I., Miller-Schlyer, M., Bigelow, D. J. and Schoneich, C. (1997). In vivo aging of rat skeletal muscle sarcoplasmic reticulum Ca^{2+}-ATPase. Chemical analysis and quantitative simulation by exposure to low levels of peroxyl radicals. *Biochim. Biophys. Acta* **1329**, 321-335.

Wahl, M. A., Koopmann, I. and Ammon, H. P. (1998). Oxidative stress causes depolarization and calcium uptake in the rat insulinoma cell RINm5F. *Exp. Clin. Endocrinol. Diabetes* **106**, 173-177.

Wang, H. and Joseph, J. A. (2000). Mechanisms of hydrogen peroxide-induced calcium dysregulation in PC12 cells. *Free Radic. Biol. Med.* **28**, 1222-1231.

Waring, P. (2005). Redox active calcium ion channels and cell death. *Arch. Biochem. Biophys.* **434**, 33-42.

Weber, H., Roesner, J. P., Nebe, B., Rychly, Y., Werner, A., Schröder, H., Jonas, L., Leitzmann, P., Schneider, K. P. and Dummler, W. (1998). Increased cytosolic Ca^{2+} amplifies oxygen radical-induced alterations of the ultrastructure and the energy metabolism of isolated rat pancreatic acinar cells. *Digestion* **59**, 175-185.

Wu, J., Takeo, T., Kamimura, N., Wada, J., Suga, S., Hoshina, Y. and Wakui, M. (1996). Thimerosal modulates the agonist-specific cytosolic Ca^{2+} oscillatory patterns in single pancreatic acinar cells of mouse. *FEBS Lett.* **390**, 149-152.

Zaidi, A., Barron, L., Sharov, V. S., Schoneich, C., Michaelis, E. K. and Michaelis, M. L. (2003). Oxidative inactivation of purified plasma membrane Ca^{2+}-ATPase by hydrogen peroxide and protection by calmodulin. *Biochemistry* **42**, 12001-12010.