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Release of Calcium from Mitochondrial and Nonmitochondrial Intracellular Stores in Mouse Pancreatic Acinar Cells by Hydrogen Peroxide

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Abstract. In the present study we have studied how $[Ca^{2+}]_i$ is influenced by H_2O_2 in collagenase-dispersed mouse pancreatic acinar cells and the mechanism underlying this effect by using a digital microspectrofluorimetric system. In the presence of normal extracellular calcium concentration, perfusion of pancreatic acinar cells with 1 mM H_2O_2 caused a slow sustained $[Ca^{2+}]_i$ increase, reaching a stable plateau after 10–15 min of perfusion. This increase induced by H_2O_2 was also observed in a nominally calcium-free medium, reflecting the release of calcium from intracellular store(s). Application of 1 mM H_2O_2 to acinar cells, in which nonmitochondrial agonist-releasable calcium pools had been previously depleted by a maximal concentration of CCK-8 (1 nM) or thapsigargin (0.5 μM) was still able to induce calcium release. Similar results were observed when thapsigargin was substituted for the mitochondrial uncoupler FCCP (0.5 μM). By contrast, simultaneous addition of thapsigargin and FCCP clearly abolished the H_2O_2-induced calcium increase. Interestingly, coincubation of intact pancreatic acinar cells with CCK-8 plus thapsigargin and FCCP in the presence of H_2O_2 did not significantly affect the transient calcium spike induced by the depletion of nonmitochondrial and mitochondrial agonist-releasable calcium pools, but was followed by a sustained increase of $[Ca^{2+}]_i$. In addition, H_2O_2 was able to block calcium efflux evoked by CCK and thapsigargin. Finally, the transient increase in $[Ca^{2+}]_i$ induced by H_2O_2 was abolished by an addition of 2 mM dithiothreitol (DTT), a sulfhydryl reducing agent. Our results show that H_2O_2 releases calcium from CCK-8- and thapsigargin-sensitive intracellular stores and from mitochondria. The action of H_2O_2 is likely mediated by oxidation of sulfhydryl groups of calcium-ATPases.

Key words: Hydrogen peroxide — Pancreatic acinar cells — Intracellular calcium stores — Sulfhydryl reagent

Introduction

Cytosolic calcium concentration ($[Ca^{2+}]_i$) is a key regulatory factor for a large number of cellular processes such as muscle contraction, metabolism, secretion or even cell differentiation and apoptosis. Numerous hormones, neurotransmitters and growth factors activate the plasma membrane phospholipase C (PLC), which generates inositol-1,4,5-triphosphate (IP_3) and diacylglycerol. IP_3, in turn releases calcium from intracellular pools, thereby initiating the calcium signal. Traditionally, it has been thought that the intracellular pools of calcium are located in the endoplasmic reticulum and in the mitochondria (Carafoli, 1987). During calcium mobilization $[Ca^{2+}]_i$ is reduced to resting level by reuptake into internal pools (Camello et al., 1996) and calcium extrusion through a plasma membrane calcium-ATPase pump (PMCA) (Tepikin et al., 1992). The release of calcium from intracellular pools also activates calcium entry across the plasma membrane (Putney, 1988).

Reactive oxygen species, such as hydrogen peroxide (H_2O_2), are known to be mediators in alteration of normal calcium homeostasis, which precede other morphological and functional alterations of cell injury induced by oxygen free radicals. Although H_2O_2 is not itself a free radical, it can be reduced to the hydroxyl radical (·OH), which is the most toxic of the oxygen-based radicals. An elevation of $[Ca^{2+}]_i$ evoked by H_2O_2 has been shown in a variety of cell types such as smooth (Roveri

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et al., 1992; Krippeit-Drews et al., 1995) and skeletal (Favero, Zable & Abramson, 1995) muscle cells, endothelial cells (Doan et al., 1994; Hu et al., 1998), mesangial cells (Meyer et al., 1996), blood mononuclear cells (Korzets et al., 1999), pancreatic β-cells (Krippeit-Drews et al., 1999), neuronal cells (Whittemore et al., 1995), cardiomyocytes (Wang et al., 1999) and renal tubular cells (Ueda & Shah, 1992). This H_2O_2-induced $[Ca^{2+}]_i$ rise has been attributed to mobilization from intracellular stores (Ueda & Shah, 1992; Wang et al., 1999), to influx across plasma membrane (Meyer et al., 1996; Korzets et al., 1999) or to both mechanisms (Roveri et al., 1992; Doan et al., 1994; Krippeit-Drews et al., 1995). Furthermore, previous studies in muscle tissue have ruled out the possibility that calcium mobilization induced by H_2O_2 is the result of an inhibition of the sarcoplasmic reticulum Ca^{2+}-ATPase pump (Castilho et al., 1996; Moreau et al., 1998) and/or an activation of the calcium release channel (Favero et al., 1995; Oba, Ishikawa & Yamaguchi, 1998).

However, the effects of H_2O_2 or other reactive oxygen species on calcium mobilization in exocrine cells have only been evaluated in a few studies. In rat pancreatic acinar cells it has been reported that hydroxyl radicals, generated by hypoxanthine/xanthine oxidase, release calcium (Weber et al., 1998) from thapsigargin-insensitive, ryanodine-sensitive calcium stores (Klonowski-Stumpe et al., 1997). More recently, it has been shown in pancreatic acinar cells that the oxidant *tert*-butylhydroperoxide disrupts repetitive calcium spiking in response to carbachol, leading to a sustained increase in $[Ca^{2+}]_i$ (Sweiry et al., 1999).

In the present paper we have studied how $[Ca^{2+}]_i$ is influenced by H_2O_2 in collagenase-dispersed mouse pancreatic acinar cells and the mechanism underlying this effect by using a digital microspectrofluorimetric system.

Material and Methods

Preparation of Acinar Cells

A suspension of single cells and small acini was prepared from mouse pancreas by enzymatic dispersion. Briefly, after cervical dislocation, the pancreas was quickly removed and placed in physiological solution containing (mM): NaCl 140, KCl 4.7, $CaCl_2$ 2, $MgCl_2$ 1.1, glucose 10 and N-2-hydroxyethylpiperazine-N′-2-sulfonic acid (HEPES) 10, pH 7.4. During isolation procedure 0.1% soybean trypsin inhibitor (Sigma) was added. Once fatty tissue and big blood vessels were removed, the pancreas was injected with a small volume (1 ml) of collagenase solution (Worthington, 200 U/ml), placed in an Eppendorf vial (1.5 ml) and incubated at 37°C under gentle agitation (approx 60 cycles/min) during 6–12 min. At the end of that period the "supernatant" was discarded and replaced with 1 ml of fresh physiological solution (same composition as before) and the vial was agitated vigorously until the cells were released to the medium. The supernatant containing a mixture of single cells and acini was transferred to a plastic test tube. The medium was replaced with another volume of solution and the procedure was repeated several times (usually 4–6). The solution with the cells was pipetted 100–150 times with a 1 ml fire-polished plastic tip and washed twice to remove cellular debris. In experiments where calcium-free medium is indicated, calcium was omitted and 2 mM EGTA was added. Cell viability monitored with trypan blue, was always greater than 95% and this was not significantly reduced by H_2O_2, at least during the duration of our experiments.

Cell Loading and $[Ca^{2+}]_i$ Determination

After isolation, the cells were suspended in physiological solution (same composition as before) and loaded with the fluorescent ratiometric calcium indicator Fura-2 by incubation with Fura-2 AM (1–2 μM, 30 min, room temperature). Once loaded, the cells were washed and used within the next 2–4 hours.

For experiments a small volume of cell suspension was placed on a thin glass coverslip attached to a Perspex perfusion chamber. Perfusion (approx. 1.5 ml/min) 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. The chamber was placed on the stage of an inverted fluorescence microscope (Nikon Diaphot 200). Cells were alternatively excited at 340 and 380 nm by a computer-controlled filterwheel (Lambda-2, Sutter Instruments) and the emitted images (>515 nm) were captured by a high speed cooled digital CCD camera (C-4880-81, Hamamatsu Photonics) and recorded using dedicated software (Argus-HiSCa, Hamamatsu Photonics).

$[Ca^{2+}]_o$ Determination

Two-milliliter aliquots of the cell suspension were placed in quartz cuvettes in a Shimadzu RF-5001PC spectrofluorimeter in the presence of 0.1 μM Calcium Green-5N (hexapotassium salt), continuously stirred and kept at a constant temperature of 37°C. Samples were excited at 506 nm and resulting fluorescence was measured at 530 nm. Either CCK-8 (1 nM), thapsigargin (0.5 μM) or H_2O_2 (1 mm) was added directly to cell suspensions in the cuvette containing nominally low calcium.

Materials

Chemicals were purchased from Sigma (Spain) except collagenase CLSPA, which was obtained from Worthington Biochemical Corporation (USA), Fura-2 AM and Calcium Green-5N (hexapotassium salt) from Molecular Probes Europe (Netherlands), thapsigargin from Alomone (Israel), and xestospongin C from Calbiochem (Spain).

Results

In the presence of normal extracellular calcium concentration, perfusion of pancreatic acinar cells with 1 mM H_2O_2 caused a slow and sustained $[Ca^{2+}]_i$ increase, which reached a stable $[Ca^{2+}]_i$ plateau after 10–15 min of perfusion (Fig. 1*A*). Figure 1*A* also shows that H_2O_2 caused a biphasic increase in $[Ca^{2+}]_i$, consisting of an initial rise observed within 3–5 min and a second sustained rise during the course of experiments. A significant proportion of the studied cells exhibited this biphasic transient increase in $[Ca^{2+}]_i$ (26 of 47 cells examined

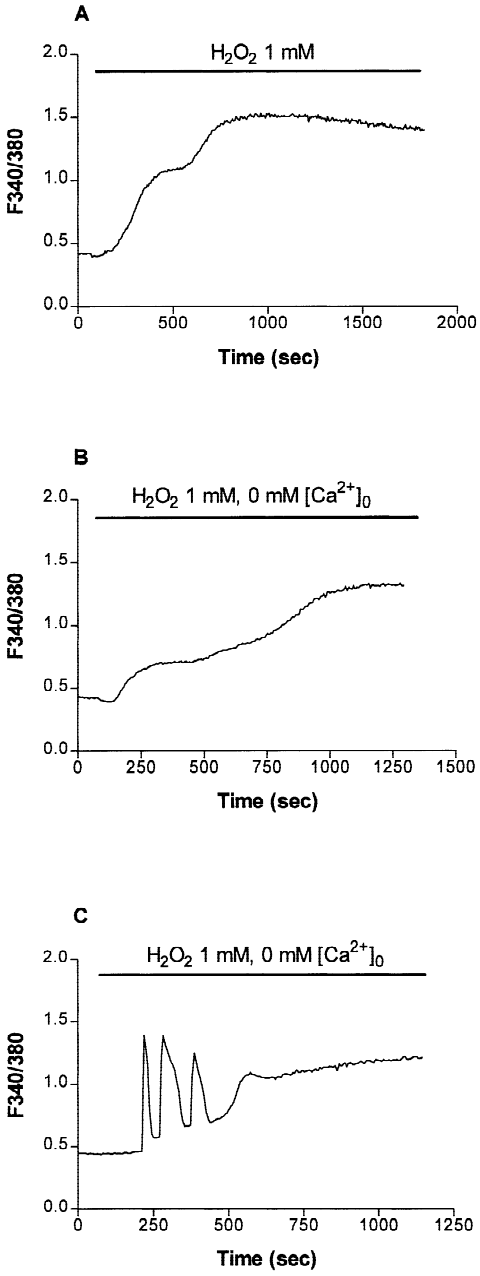

Fig. 1. Mobilization of calcium in response to H_2O_2 in pancreatic acinar cells. Cells were perfused with 1 mM H_2O_2 in calcium-normal (*A*) or -free (*B* and *C*) solution. Traces are representative of 5–9 independent experiments.

from 5 experiments, 55%). Figure 1*B* demonstrates that the increase of $[Ca^{2+}]_i$ induced by H_2O_2 was also observed in calcium-free medium, reflecting the release of calcium from intracellular store(s). In some cells H_2O_2 induced a few repetitive calcium spikes, followed by an increase to a new constant level, even in the absence of extracellular calcium (15 of 70 studied cells from 9 experiments, 22%) (Fig. 1*C*).

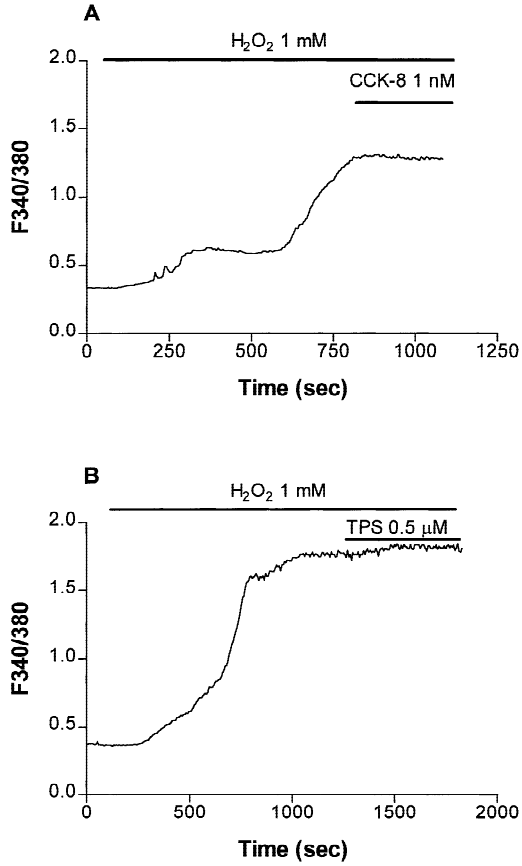

Fig. 2. Mobilization of calcium in response to agonists in pancreatic acinar cells pretreated with H_2O_2. Cells were initially perfused with 1 mM H_2O_2 followed by 1 nM CCK-8 (*A*) or 0.5 μM thapsigargin (TPS) (*B*) in calcium-free solution. Traces are representative of 3–4 independent experiments.

Pretreatment of cells with H_2O_2 abolished both the typical calcium response both to CCK-8 (Fig. 2*A*) and thapsigargin (Fig. 2*B*) in the absence of extracellular calcium. However, when the nonmitochondrial agonist-releasable calcium pools had been previously depleted by a maximal concentration (1 nM) CCK-8 or 0.5 μM thapsigargin in a calcium-free solution, 1 mM H_2O_2 was still able to induce $[Ca^{2+}]_i$ increases in 41 of 51 cells recorded (80%) from 6 experiments (Fig. 3*A* and *B*). Similar results were observed when thapsigargin was substituted for FCCP, a mitochondrial uncoupler that collapses the mitochondrial membrane potential and calcium uptake into the mitochondria driven by the membrane potential. As shown in Fig. 3*C*, pretreatment of the acinar cells with 0.5 μM FCCP was also unable to suppress the increase in $[Ca^{2+}]_i$ induced by H_2O_2 in a calcium-free solution (21 of 27 cells examined from 5 experiments). By contrast, simultaneous addition of thapsigargin and FCCP (which depleted nonmitochondrial intracellular calcium stores, e.g., endoplasmic reticulum and mitochondria) clearly abolished the H_2O_2-

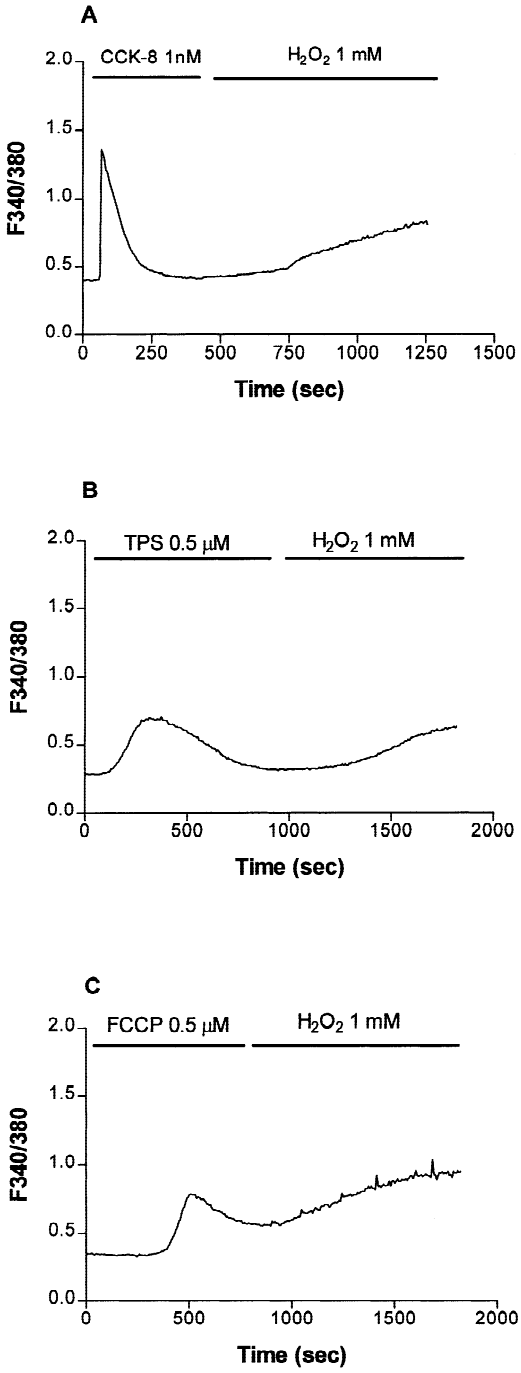

Fig. 3. Effect of agonist-induced calcium store depletion on H_2O_2-evoked calcium increase. Pancreatic acinar cells were perfused with either 1 nM CCK-8 (*A*), 0.5 μM thapsigargin (TPS) (*B*) or 0.5 μM FCCP (*C*) in calcium-free solution, followed by 1 mM H_2O_2. Traces are representative of 5–6 independent experiments.

induced calcium increase in all 22 cells examined from 4 experiments (Fig. 4), suggesting that H_2O_2 releases calcium from both mitochondrial and nonmitochondrial calcium pools. Since it had been previously shown that the

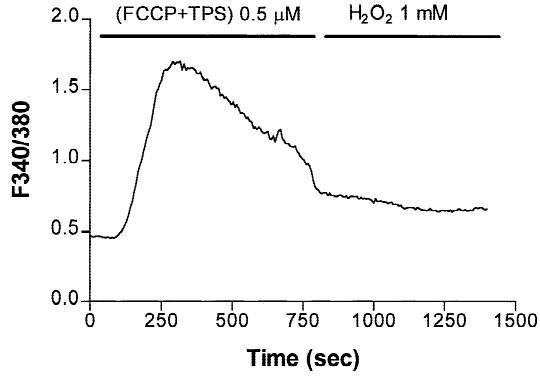

Fig. 4. Effect of thapsigargin plus FCCP on H_2O_2-evoked calcium increase. Pancreatic acinar cells were co-incubated with 0.5 μM thapsigargin (TPS) plus 0.5 μM FCCP followed by 1 mM H_2O_2 in calcium-free solution. Trace is representative of 4 independent experiments.

sulfhydryl reagent thimerosal is able to sensitize the IP_3-induced calcium release (Thorn et al., 1992; Wu et al., 1996), we also evaluated if H_2O_2 can release calcium by sensitizing the IP_3-induced calcium release. To test this possibility we employed xestospongin C, a very potent, reversible and membrane-permeable blocker of IP_3-mediated calcium release that does not interact with the IP_3-binding site (Gafni et al., 1997). As shown in Fig. 5, application of 10 μM xestospongin C to acinar cells, which mitochondrial calcium pool had been previously depleted by FCCP, was unable to suppress the increase in $[Ca^{2+}]_i$ induced by 1 mM H_2O_2. However, xestospongin C reversibly inhibited $[Ca^{2+}]_i$ oscillations evoked by 100 pM CCK-8 in 21 of 25 cells examined (84%) from 3 experiments (Fig. 6).

To know whether H_2O_2 also acts on calcium extrusion to the extracellular medium we followed the protocol shown in Fig. 7*A*. Cells were stimulated with a combination of CCK-8 and thapsigargin in calcium-free medium to release calcium from nonmitochondrial stores and to block calcium reuptake into the stores. In these conditions $[Ca^{2+}]_i$ decay is driven by PMCA activity (this cell type has no significant Na^+/Ca^{2+} exchange across plasma membrane (Muallem, Beeker & Pandol, 1988)). As shown in Fig. 7*A* co-incubation with H_2O_2 induced a secondary $[Ca^{2+}]_i$ increase followed by a prolonged plateau. This can be due to either calcium release from mitochondria (they accumulate calcium during calcium mobilization — Camello, Pariente & Camello, *unpublished observations*-) or impairment of PMCA activity. So, we performed the same experiments but adding FCCP to the combination of CCK-8 plus thapsigargin, given that FCCP rapidly collapses mitochondrial potential and releases mitochondrial calcium. The inset of Fig. 7*B* shows that this treatment leads to a slowed $[Ca^{2+}]_i$ decay without the secondary $[Ca^{2+}]_i$ increase observed in Fig. 7*A*. When H_2O_2 was added the initial peak was immediately estabilized at an elevated plateau, in-

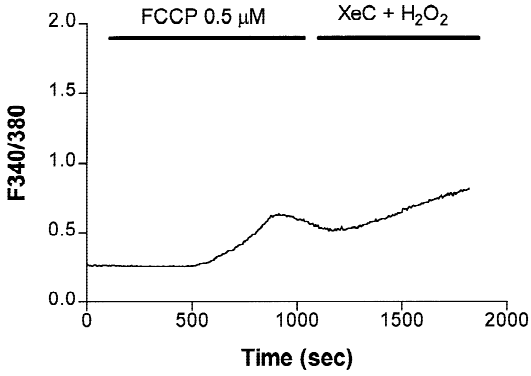

Fig. 5. Effect of the IP_3-receptors antagonist xestospongin C on H_2O_2-evoked nonmitochondrial calcium increase. Pancreatic acinar cells were perfused with 0.5 μM FCCP in calcium-free solution, followed by 10 μM xestospongin C (XeC) and 1 mM H_2O_2. Trace is representative of 4 independent experiments.

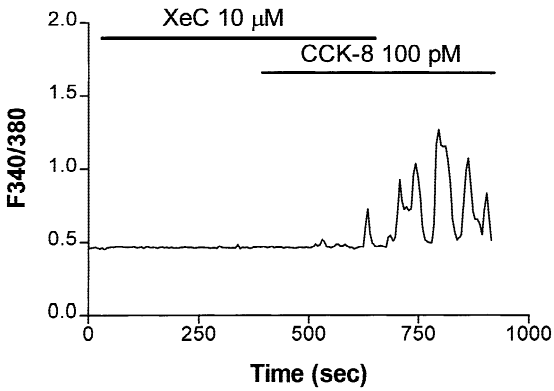

Fig. 6. Effect of the IP_3-receptors antagonist xestospongin C on calcium signaling induced by CCK-8. Pancreatic acinar cells were perfused with 10 μM xestospongin C (XeC) followed by 100 pM CCK-8 in calcium-free solution. Trace is representative of 3 independent experiments.

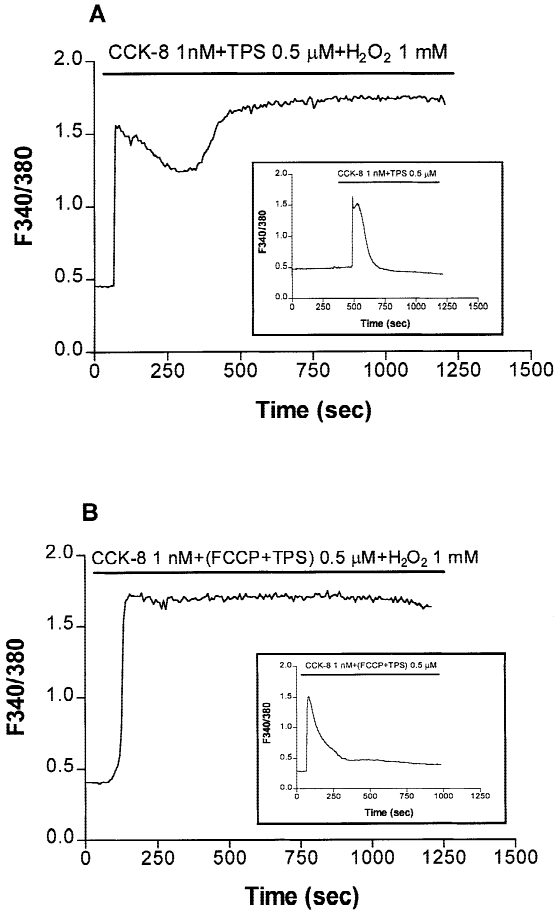

Fig. 7. Effect of H_2O_2 on the plateau phase of calcium in response to agonists. Pancreatic acinar cells were co-incubated with 1 nM CCK-8 and 0.5 μM thapsigargin (TPS) (*A* and *B*) plus 0.5 μM FCCP (*B*) in the presence of 1 mM H_2O_2 (*A* and *B*) in calcium-free solution. For comparisons, the insets show the effect of each treatment applied in the absence of H_2O_2. Traces are representative of 3–5 independent experiments.

dicating that the main action of H_2O_2 during the decay phase of $[Ca^{2+}]_i$ is an inhibition of calcium extrusion via PMCA. To further assess the effect of H_2O_2 on calcium extrusion we determined directly calcium transport from cytosol to external medium using the cell impermeant form of a low affinity calcium dye, Calcium Green 5-N in cell populations. Figure 8 shows that Calcium Green fluorescence is enhanced following stimulation of the cells with CCK-8 plus thapsigargin, reflecting transport of cytosolic calcium to the external solution. In H_2O_2-treated experiments this calcium extrusion was clearly blocked.

Finally, in the absence of the extracellular calcium, the transient increase in $[Ca^{2+}]_i$ induced by H_2O_2 application was abolished by an addition of 2 mM of the sulfhydryl reducing agent dithiothreitol (DTT) in all 18 cells examined from 3 experiments (Fig. 9*A*). This inhibition was reversible; the removal of DTT allows $[Ca^{2+}]_i$ to return to the transient increase evoked by H_2O_2. The effect of the hydroxyl radical scavenger, melatonin, was also assessed under the same experimental conditions. Application of 1 mM melatonin did not affect the increase in $[Ca^{2+}]_i$ caused by H_2O_2 (Fig. 9*B*).

Discussion

The present study has demonstrated that the reactive oxygen species H_2O_2 induces a $[Ca^{2+}]_i$ increase in pancreatic acinar cells by calcium release from intracellular stores, since it was observed in calcium-free solution. On the other hand, pretreatment of acinar cells with H_2O_2 followed by the addition of either CCK-8 or thap-

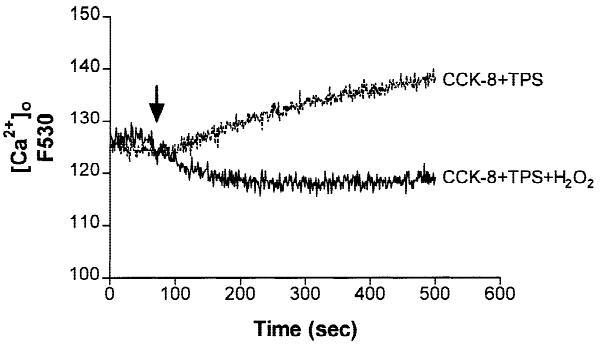

Fig. 8. Effect of H_2O_2 on release of calcium (calcium efflux) from pancreatic acinar cells evoked by CCK-8 plus thapsigargin, challenged with Calcium Green-5N hexapotassium salt (cell-impermeant form), in a nominally low-calcium medium. The arrow indicates the point of addition of each drug to the cuvette (1 nM CCK-8, 0.5 μM thapsigargin (TPS), 1 mM H_2O_2). Traces are representative of 3 independent experiments.

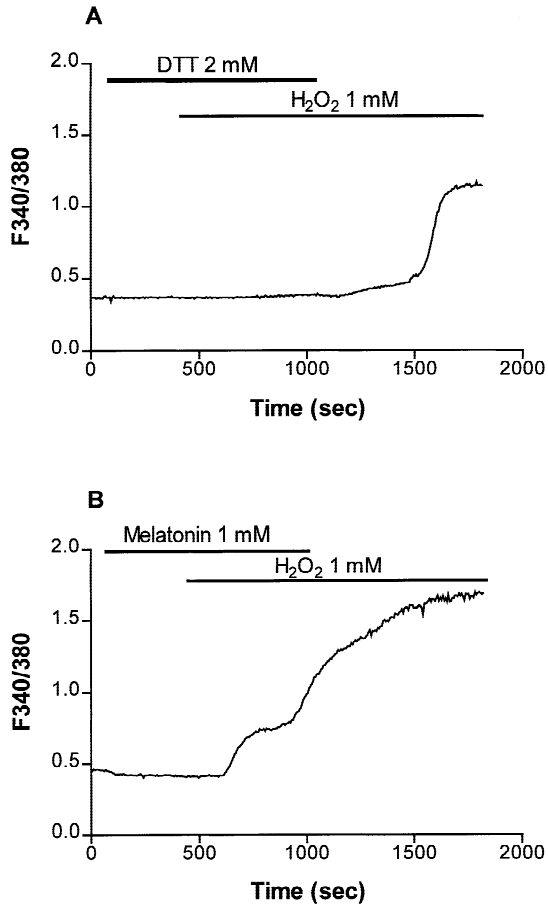

Fig. 9. Effect of either dithiothreitol (DTT) or melatonin on H_2O_2-evoked calcium mobilization. Pancreatic acinar cells were perfused with either 2 mM DTT (*A*) or 2 mM melatonin (*B*) followed by 1 mM H_2O_2 in calcium-free solution. Traces are representative of 3–4 independent experiments.

sigargin resulted in a reduction in the agonist-evoked rise in $[Ca^{2+}]_i$, whereas H_2O_2 failed to increase $[Ca^{2+}]_i$ in cells which nonmitochondrial and mitochondrial intracellular calcium stores had been previously depleted by application of thapsigargin and FCCP, in calcium-free solution. In addition, we present evidence that H_2O_2 acts by a mechanism that involves sulfhydryl group oxidation.

In pancreatic acinar cells, the stimulatory effect of H_2O_2 on resting $[Ca^{2+}]_i$ and its inhibitory effect on agonist-induced calcium mobilization could be due to a direct effect on the calcium release process and not a consequence of the opposing action in the calcium pathway. Previous studies in different cell types, including endothelial cells (Doan et al., 1994), mouse oocytes (Carroll & Swann, 1992), HeLa cells (Bootman, Taylor & Berridge, 1992) and hepatocytes (Bird, Burgess & Putney, 1993), have reported that hydroperoxides and other sulfhydryl reagents can induce calcium mobilization. In pancreatic acinar cells, we and others have previously shown that the sulfhydryl group oxidizing agents thimerosal (Thorn et al., 1992), vanadate (Pariente et al., 1999) and phenylarsine oxide (Lajas et al., 1999) are able to mobilize calcium from intracellular stores, and are reversible in the presence of the thiol-reducing agent dithiothreitol (DTT). Our results, demonstrating that H_2O_2 releases calcium from intracellular stores, suggest that the failure of CCK-8 and thapsigargin to induce calcium mobilization after H_2O_2 is related to a partial or complete depletion of the stores by this agent. The H_2O_2-sensitive calcium pool includes those released by thapsigargin (e.g., endoplasmic reticulum) and FCCP (e.g., mitochondria). This is shown by the failure of H_2O_2 to increase $[Ca^{2+}]_i$ after treatment with thapsigargin plus FCCP in calcium-free medium. Thus, when the nonmitochondrial agonist-releasable calcium pools are previously depleted by CCK-8 or thapsigargin, H_2O_2 is able to induce calcium release from mitochondria in a calcium-free medium, whereas if the mitochondrial calcium is released by treatment with FCCP, H_2O_2 releases the calcium from thapsigargin-sensitive pool. In this context, it is important to note that the existence of two major types of intracellular calcium stores has been suggested: (i) the endoplasmic reticulum, which functions as a high-affinity, low-capacity calcium pool, and (ii) mitochondria, which are low-affinity, high-capacity calcium pools (Carafoli, 1987). These two intracellular calcium pools could also explain the biphasic transient increase in $[Ca^{2+}]_i$ induced by H_2O_2 in some of our cells. One of the two rises in $[Ca^{2+}]_i$ could be due to mobilization of calcium from endoplasmic reticulum/mitochondria (and vice versa). The initial phase is due to release of nonmitochondrial calcium, and the second is due to release of mitochondrial calcium. This observation is also substantiated by the significant delay in the release of calcium

from the mitochondrial pool compared to release from the nonmitochondrial pool (Fig. 3) (374 ± 37 sec, 21 cells for FCCP experiments versus 105 ± 14 sec, 28 cells for thapsigargin experiments). It is also worth noting that once the nonmitochondrial pool is depleted, H_2O_2 causes a slow calcium release (corresponding to the mitochondrial store) (Fig. 3*A* and *B*), while when the mitochondrial store is initially depleted the H_2O_2 effect is much faster (Fig. 3*C*), as expected if associated to release from the nonmitochondrial pool.

Our findings, in which H_2O_2 releases calcium from intracellular stores, are consistent with previous reports, where the $[Ca^{2+}]_i$ increase evoked by H_2O_2 is accomplished by an inhibition of the Ca^{2+}-ATPase pump (Castilho et al., 1996; Moreau et al., 1998) and/or by activation of the calcium release channel (Favero et al., 1995; Oba et al., 1998). In fact, it has been reported that sulfhydryl groups have a critical role in Ca^{2+}-ATPase function (Bellomo et al., 1983) and in ryanodine-sensitive calcium channel (Oba et al., 1998). Additionally, in pancreatic acinar cells it has been shown that the sulfhydryl group oxidizing agent thimerosal is able to mobilize calcium from intracellular stores by sensitizing the IP_3 receptor to the endogenous level of IP_3 (Thorn et al., 1992). In fact, ryanodine and IP_3 receptors show "in vitro" sensitization in the presence of thimerosal (Abramson et al., 1995; Wu et al., 1996). However, our results using the recently developed membrane-permeable IP_3-receptor blocker xestospongin C demonstrate the H_2O_2 releases calcium from a nonmitochondrial calcium pool by an IP_3-receptors independent mechanism.

The results of this study have also shown that stimulation of cells with a combination of H_2O_2 with CCK-8, thapsigargin and FCCP resulted in a potentiation of the plateau phase of calcium response to these agents without modifying the transient calcium spike. This observation is in agreement with previous studies in pancreatic acinar cells (Hirohata et al., 1998; Pariente et al., 1999) and in a thyroid cell line (Meucci et al., 1995), where vanadate, another sulfhydryl agent, enhanced the plateau phase of the calcium response to agonists. Similar results have been observed by us using phenylarsine oxide and dephostatin, other sulfhydryl reagent (Lajas et al., 1999; 2000). This stimulatory effect of H_2O_2 on the plateau phase of the calcium response to agonists is due to the blockade of calcium extrusion mechanism by the acinar cell plasma membrane, as indicated by the finding that H_2O_2 blocks the calcium efflux to the extracellular medium evoked by CCK-8 plus thapsigargin. In fact, H_2O_2 has been shown to be an inhibitor of the plasma membrane Ca^{2+}-ATPase pump in neuronal membrane (Zaidi & Michaelis, 1999). Taken together, our observations suggest that H_2O_2 inhibits both Ca^{2+}-ATPase involved in the intracellular sequestration of calcium within the stores in the acinar cells (acting as a thapsigargin-like agent) (one of the mechanism probably involved in the effects of Figs. 1 and 2) and the plasma membrane Ca^{2+}-ATPase pump (the mechanism probably involved in Figs. 7 and 8), thereby increasing $[Ca^{2+}]_i$. Additionally, H_2O_2 can also release calcium from the mitochondria. The release of calcium from mitochondria can occur by oxidation of thiol groups in membrane proteins (Chakraborti et al., 1999).

The effects of H_2O_2 on calcium mobilization were strongly blocked by the presence of the sulfhydryl reducing agent DTT. It has been previously shown that sulfhydryl-oxidizing reagents mobilize calcium in different cell types (Fletcher, Samelson & June, 1993; Fleming, Bara & Busse, 1996), including pancreatic acinar cells (Thorn et al., 1992; Lajas et al., 1999). Furthermore, it is known that sulfhydryl groups play a critical role on Ca^{2+}-ATPase function and that sulfhydryl-oxidizing reagents markedly inhibit ATP-dependent calcium uptake in liver plasma membrane fractions (Bellomo et al., 1983). Our results, taken together with the known effects of H_2O_2 as an oxidizing agent, suggest that the effects of H_2O_2 on calcium mobilization are mediated by a mechanism that involves sulfhydryl group oxidation. Thus, the reduced form of DTT, which protects sulfhydryl groups from oxidation, blocked the effects of H_2O_2, presumably by reducing sulfhydryl groups.

Finally, pretreatment with the hydroxyl radical scavenger melatonin (Tan et al. 1993) was ineffective on the H_2O_2-induced calcium increase, indicating that an increase in the formation of the highly toxic hydroxyl radicals (formed from H_2O_2) is not likely to be the main mechanism of action of H_2O_2 on calcium mobilization, despite its known activity as oxygen free radical.

In summary, our findings show that the treatment of acinar cells with H_2O_2 results in the release of calcium from mitochondrial and nonmitochondrial intracellular calcium stores. This effects of H_2O_2 could be blocked by the use of DTT, indicating that its action is likely mediated by oxidation of sulfhydryl groups of Ca^{2+}-ATPases. From a physiological point of view, taken together, the results of our study help us to understand the complex mechanism of calcium homeostasis in pancreatic acinar cells and provide evidence that H_2O_2 could be used as a pharmacological tool in calcium mobilization.

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