Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 491–502
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Effect of hydrogen peroxide on Ca^{2+} mobilisation in human platelets through sulphydryl oxidation dependent and independent mechanisms

Pedro C. Redondo, Ginés M. Salido, Juan A. Rosado*, José A. Pariente

Department of Physiology, University of Extremadura, Av. Universidad s/n, 10071 Caceres, Spain

Received 6 June 2003; accepted 26 September 2003

Abstract

Using Fura-2-loaded human platelets we studied the nature of the mechanisms involved in Ca^{2+} signalling mediated by H_2O_2. In a Ca^{2+}-free medium, H_2O_2 (10 μM–100 mM) induced a concentration-dependent increase in $[Ca^{2+}]_i$. Depletion of either agonist-sensitive or mitochondrial Ca^{2+} pools reduced this effect while depletion of both stores abolished it. Xestospongin C, an inositol 1,3,5-trisphosphate (IP_3) receptor inhibitor, reduced Ca^{2+} release evoked by 1 mM H_2O_2 by 45%, indicating that H_2O_2-induced Ca^{2+} release involves interaction with IP_3 receptors. Blockade of the IP_3 turnover by lithium or treatment with U-73122 did not modify H_2O_2-induced Ca^{2+} release from the agonist-sensitive pool, suggesting the involvement of a mechanism independent of IP_3 generation. H_2O_2 inhibited Ca^{2+} reuptake into the agonist-sensitive stores mediated by the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA). Thimerosal (5 μM), a sulphydryl reagent, induced Ca^{2+} release from the agonist-sensitive stores. This event was impaired by treatment with 2 mM DTT, which also inhibited H_2O_2-induced Ca^{2+} release from the agonist-sensitive pool but not from mitochondria. H_2O_2 reduced the ability of the plasma membrane Ca^{2+} ATPase (PMCA) to extrude Ca^{2+} by 75%, an effect that was unaffected by DTT. Consistent with this, thimerosal did not modify the PMCA activity. Finally, exposure to H_2O_2 triggered platelet aggregation, which was slower than that observed after agonist stimulation. We conclude that H_2O_2 induced Ca^{2+} release from agonist-sensitive stores by oxidation of sulphydryl groups in SERCA and the IP_3 receptors independently of IP_3 generation. In addition, H_2O_2 induced Ca^{2+} release from mitochondria and inhibited the PMCA activity by different mechanisms in human platelets.

Keywords: H_2O_2; Ca^{2+} release; Human platelets; IP_3 receptor; Thimerosal; SERCA

1. Introduction

Cytosolic Ca^{2+} is a key factor involved in the regulation of a large number of cellular functions. Increases in $[Ca^{2+}]_i$ can initiate and modulate many different processes, including short-term responses, such as muscle contraction or secretion and long-term events like cell growth [1]. Recently, a great deal of attention has focused on the sensitivity of the mechanisms involved in Ca^{2+} mobilisation to changes in the redox state [2–4]. Oxidative modification of Ca^{2+} channels or proteins involved in Ca^{2+} metabolism has been observed in several cell types suggesting that changes in the redox potential may play an important role in Ca^{2+} homeostasis [5,6].

In human platelets, interaction with agonists initiates the activation of several intracellular signal transduction pathways, including the elevation in $[Ca^{2+}]_i$, which are responsible for the physiological responses, such as aggregation or synthesis and secretion of biologically active molecules, which, in turn, activate resting platelets. Among these agents are ADP, thomboxane A_2 and ROS [7,8]. Several sources of ROS have been suggested in platelets and other cells, including the superoxide-dismutase, the activation of arachidonic acid metabolism, the metabolism of phosphoinositides and the activity of NADH/NADPH oxidase [8–10].

ROS have also been reported to act as second messengers involved in the activation of intracellular pathways such as arachidonic acid metabolism or phospholipase C dependent signal transduction [11,12]. Consistent with this, ROS, such as H_2O_2, mediate changes in $[Ca^{2+}]_i$ in

* Corresponding author. Tel.: +34-927-257154; fax: +34-927-257154.
E-mail address: jarosado@unex.es (J.A. Rosado).

Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; SERCA, sarcoendoplasmic reticulum Ca^{2+} ATPase; TG, thapsigargin; HBS, HEPES-buffered saline; DTT, dithiotreitol; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; ROS, reactive oxygen species; PMCA, plasma membrane Ca^{2+} ATPase; IP_3, inositol 1,3,5-trisphosphate; PI3-K, phosphatidylinositol 3-kinase.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.09.031

several cell types, which precede other morphological or functional modifications both in physiological and pathophysiological conditions [13,14]. H_2O_2-induced $[Ca^{2+}]_i$ elevation has been mainly attributed to Ca^{2+} release from agonist releasable stores or a mitochondrial Ca^{2+} pool [13–15]. Ca^{2+} release from agonist-sensitive stores might be mediated by either inhibition of SERCA [16] or by activation of Ca^{2+} release channels [13,14,17]. However, although the effect of ROS on ryanodine receptors is well established to occur through the oxidation of hyperreactive sulphydryl groups [18], the nature of the mechanism involved in H_2O_2-induced Ca^{2+} release by interaction with IP_3 receptors or SERCA remains unclear. In addition, H_2O_2 might modify $[Ca^{2+}]_i$ by interacting with the PMCA, the main mechanism involved in Ca^{2+} extrusion in human platelets [19], although its effect on PMCA activity is controversial.

In the present study we sought to expand our understanding of the mechanisms underlying ROS-induced Ca^{2+} mobilisation in non-excitable cells, such as human platelets, which lack of ryanodine receptors and where the PMCA is the major pump for Ca^{2+} extrusion, and therefore is an excellent model to investigate the effect of oxidation on IP_3 receptors and Ca^{2+} extrusion mediated by the PMCA.

2. Materials and methods

2.1. Materials

Fura-2 acetoxymethyl ester (Fura-2/AM) and BCECF/AM were from Molecular Probes. Bovine serum albumin (BSA), apyrase (grade VII), aspirin, thrombin, H_2O_2, lanthanum chloride, FCCP, thimerosal, lithium chloride, catalase, oligomycin, EGTA, U-73122, wortmannin, dimethyl BAPTA and TG were from Sigma. Ionomycin, rotenone and xestospongin C were from Calbiochem. DTT was purchased from Bio-Rad. Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology. Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) was from Amersham. All other reagents were purchased from Panreac.

2.2. Platelet preparation

Fura-2-loaded platelets were prepared as described previously [20]. Briefly, blood was drawn by venepuncture from healthy drug-free volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing 85 mM sodium citrate, 78 mM citric acid and 111 mM D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700 *g* and aspirin (100 μM) and apyrase (40 μg/mL) added. Platelet-rich plasma was incubated at 37° with 2 μM Fura-2/AM for 45 min. Cells were then collected by centrifugation at 350 *g* for 20 min and resuspended in HBS containing 145 mM NaCl, 10 mM HEPES, 10 mM D-glucose, 5 mM KCl, 1 mM $MgSO_4$, pH 7.45 and supplemented with 0.1% (w/v) BSA and 40 μg/mL apyrase.

Cell viability was assessed using tripan blue and BCECF. Cellular viability after treatment with 1 mM H_2O_2, monitored with the tripan blue exclusion technique, was 94%, similar to that observed in a resting platelet suspension, at least during the performance of the experiments. For BCECF loading, cells were incubated for 30 min with 5 μM BCECF-AM at 37°. Resting platelets and platelets treated with 1 mM H_2O_2 for 12 min were centrifuged and both the pellet (resuspended in fresh HBS) and the supernatant were collected. Fluorescence was recorded from 2 mL aliquots using a Shimadzu Spectrophotometer. Samples were excited at 488 nm, and the isosbestic wavelength for pH changes, 440 nm, and the resulting fluorescence was measured at 535 nm. The results obtained with BCECF confirm those observed using tripan blue. After treatment for 12 min with 1 mM H_2O_2 the BCECF fluorescence in the cells was 93 and 100% of control when the samples were excited with 488 and 440 nm, respectively, suggesting that under our conditions there was not severe cellular damage. The reduction in cellular BCECF fluorescence was accompanied by a proportional increase in the supernatant (data not shown).

2.3. Measurement of $[Ca^{2+}]_i$

Fluorescence was recorded from 2 mL aliquots of magnetically stirred platelet suspension (10^8 cells/mL) at 37° using a Shimadzu Spectrophotometer. Samples were alternatively excited at 340 and 380 nm and the resulting fluorescence was measured at 505 nm. Changes in $[Ca^{2+}]_i$ were monitored using the Fura-2 340/380 nm fluorescence ratio and calibrated according to the method of Grynkiewicz *et al.* [21].

Ca^{2+} release from internal stores after platelet stimulation in a Ca^{2+}-free medium was estimated as the integral of the rise in $[Ca^{2+}]_i$ above basal for the time indicated after addition of the agents.

To compare the rate of decay of $[Ca^{2+}]_i$ to basal values, after treatment of platelets with thrombin or TG plus ionomycin, in the absence or presence of different agents we used the constant of the exponential decay as previously described [19]. Traces were fitted to the equation: $y = A(1 - e^{-K_1 T})\, e^{-K_2 T}$, where K_1 and K_2 are the constants of the exponential increase and decay, respectively, T is time and A is the span.

H_2O_2 did not modify the Fura-2 fluorescence as checked with Fura-2 pentapotassium salt.

2.4. Protein tyrosine phosphorylation

Protein tyrosine phosphorylation was detected by gel electrophoresis and Western blotting [19]. Platelets stimu-

lation was terminated by mixing with an equal volume of two times Laemmli's buffer with 10% dithiothreitol followed by heating for 5 min at 95°. One-dimensional SDS-electrophoresis was performed with 10% polyacrylamide minigels and separated proteins were electrophoretically transferred, for 2 hr at 0.8 mA/cm^2, in a semi-dry blotter (Hoefer Scientific) onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in Tris-buffered saline with 0.1% Tween-20 (TBST) to block residual protein binding sites. Immunodetection of tyrosine phosphorylation was achieved using the anti-phosphotyrosine antibody 4G10 diluted 1:1500 in TBST for 1 hr. The primary antibody was removed and blots washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with horseradish peroxidase-conjugated ovine anti-mouse IgG antibody diluted 1:10,000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic films and the integrated optical density of the blots was estimated using scanning densitometry.

For loading with dimethyl BAPTA, cells were incubated for 30 min at 37° with 10 μM dimethyl BAPTA/AM. Cells were then collected by centrifugation and resuspended in HBS as described above.

2.5. Platelet aggregation

Platelet aggregation was performed in platelet-rich plasma, prepared as described previously. Platelet counts were normalised to 2×10^8 cells/mL. Calcium (1 mM) was then added to the platelet suspension and aggregation was performed using a Chronolog lumiaggregometer as described elsewhere [22] and expressed as percentage [22].

2.6. Statistical analysis

Analysis of statistical significance was performed using Student's *t*-test and only values with $P < 0.05$ were accepted as significant.

3. Results

3.1. H_2O_2 releases Ca^{2+} from agonist releasable and mitochondrial Ca^{2+} stores

In a Ca^{2+}-free medium (200 μM EGTA was added), treatment of human platelets with H_2O_2 resulted in a concentration-dependent increase in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from intracellular stores (Fig. 1A). Ca^{2+} release was detected at 10 μM H_2O_2 with a 13.5 ± 2.6% of the maximal stimulation, and the half-maximal effect (EC_{50}) was found at 1.63 mM, therefore 1 mM was the concentration used throughout this study. Figure 1B and D show representative experiments with 1 mM H_2O_2, which induces a slow and sustained increase in $[Ca^{2+}]_i$ that reaches a stable $[Ca^{2+}]_i$ plateau after 2–5 min of treatment. Pretreatment of platelets with 1 mM H_2O_2 significantly reduced the typical Ca^{2+} release stimulated by the physiological agonist thrombin (1 U/mL, Fig. 1B compared to E; $P < 0.05$, N = 8) or by treatment with 1 μM TG, a specific inhibitor of SERCA [23] plus a low concentration of ionomycin (50 nM; required for extensive depletion of the intracellular Ca^{2+} stores in platelets where two agonist-releasable Ca^{2+} stores with high and low Ca^{2+} leakage rates have been reported [24]; Fig. 1D compared to F; $P < 0.05$, N = 8), suggesting that H_2O_2 releases Ca^{2+} from agonist-sensitive stores. Similar results were obtained with thimerosal, a sulphydryl reagent. Treatment of platelets with 5 μM thimerosal significantly reduced thrombin-induced Ca^{2+} release (Fig. 1C; $P < 0.05$, N = 5). It is noteworthy that complete depletion of agonist-sensitive Ca^{2+} stores by treatment with 1 U/mL thrombin or 1 μM TG plus 50 nM ionomycin was unable to prevent H_2O_2-induced Ca^{2+} release, suggesting that H_2O_2 is also able to release Ca^{2+} from an agonist-insensitive store (Fig. 1E and F).

In order to investigate the nature of the H_2O_2 releasable agonist-insensitive Ca^{2+} store we use FCCP, a mitochondrial uncoupler that collapses the mitochondrial membrane potential that drives Ca^{2+} uptake [25]. As shown in Fig. 2A, treatment of human platelets with 1 μM FCCP in a Ca^{2+}-free medium resulted in a sustained increase in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from mitochondrial stores. Subsequent addition of 1 mM H_2O_2 to the platelet suspension was still able to release Ca^{2+}, presumably from agonist-sensitive stores. Treatment with H_2O_2 reduced Ca^{2+} release from mitochondria evoked by subsequent addition of FCCP (Fig. 2B), suggesting that mitochondrial stores are partially depleted by pretreatment with H_2O_2. Similar results were obtained when mitochondrial Ca^{2+} uptake was prevented using 10 μM rotenone, an inhibitor of complex I of the respiratory chain that dissipates the membrane potential [26], plus 10 μM oligomycin, a specific inhibitor of the membrane-bound mitochondrial ATP-synthase (F_1) [27], added to prevent ATP depletion by hydrolysis of ATP after mitochondrial membrane potential collapse (data not shown).

By contrast, simultaneous depletion of agonist-sensitive stores using thrombin or TG plus ionomycin and mitochondrial stores by FCCP or oligomycin plus rotenone clearly abolished H_2O_2-induced Ca^{2+} release (Fig. 2C–E; N = 6). These findings indicate that H_2O_2 releases Ca^{2+} from both agonist-sensitive and mitochondrial Ca^{2+} pools.

3.2. H_2O_2-induced release of Ca^{2+} from agonist-sensitive stores is mediated by oxidation of the IP_3 receptor and SERCA

A number of studies have proposed that modulation of the redox potential might be a general mechanism to

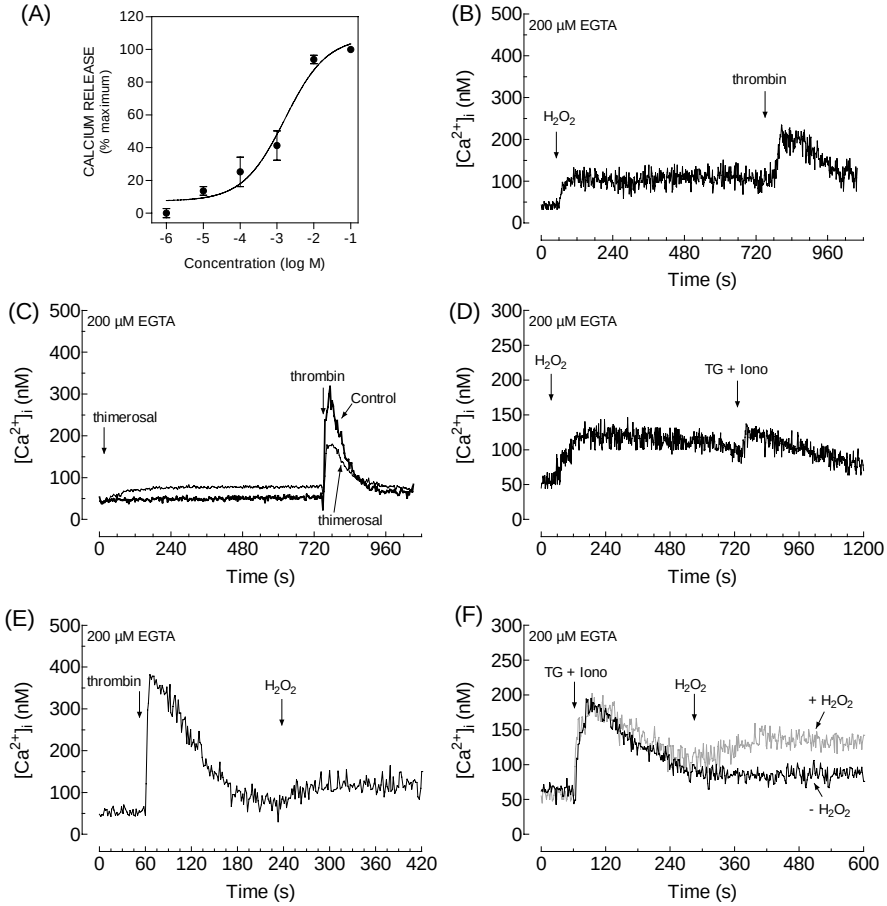

Fig. 1. Hydrogen peroxide releases Ca^{2+} from agonist-sensitive and -insensitive stores in human platelets. Human platelets were loaded with Fura-2 and resuspended in a Ca^{2+}-free medium as described in the Methods section. Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_i$. At the time of experiment 200 μM EGTA was added. (A) Fura-2-loaded human platelets were treated with various concentrations (1 μM–100 mM) of H_2O_2 and Ca^{2+} release was calculated as the integral for 6 min of the rise in $[Ca^{2+}]_i$. Values are mean ± SEM, N = 4. (B–D) Cells were treated with 1 mM H_2O_2 (B and D) or 5 μM thimerosal (C) and 12 min later 1 U/mL thrombin (B and C) or 1 μM TG plus 50 nM ionomycin (Iono; D) were added to the platelets suspension. (E) Fura-2-loaded platelets were stimulated with 1 U/mL thrombin and 3 min later 1 mM H_2O_2 was added. (F) Cells were treated with 1 μM TG plus 50 nM ionomycin and 3 min later 1 mM H_2O_2 ($+H_2O_2$) or the vehicle ($-H_2O_2$) were added. The traces shown are representative of 4–10 separate experiments.

regulate intracellular Ca^{2+} homeostasis. Hence, we have investigated whether the effect of H_2O_2 on agonist-releasable stores might be mediated by interaction with the IP_3 receptor function. To test this possibility human platelets were preincubated with the cell permeant inhibitor of the IP_3 receptor function xestospongin C [28]. To avoid interference with mitochondrial Ca^{2+} release rotenone plus oligomycin were added to the buffer to deplete the mitochondrial pool. Xestospongin C had no detectable effects either on Ca^{2+} release or TG-induced Ca^{2+} release (Fig. 3A, inset; N = 3). Pretreatment for 30 min with xestospongin C (20 μM) significantly reduced H_2O_2-induced Ca^{2+} release from agonist-sensitive stores by 45.5 ± 5.6% as estimated by the integral of the rise in $[Ca^{2+}]_i$ above basal for 6 min after the addition of H_2O_2 (Fig. 3A; $P < 0.05$, N = 14). This effect was subsequently demonstrated by testing the amount of Ca^{2+} remaining in the stores using TG plus ionomycin. In cells pretreated with xestospongin C, addition of 1 μM TG plus 50 nM ionomycin after H_2O_2 induced a significantly higher Ca^{2+} release (145.8 ± 12.6% of control as estimated by the integral of the rise in $[Ca^{2+}]_i$ above basal for 2.5 min after the addition of TG plus ionomycin) indicating that under these conditions H_2O_2-induced Ca^{2+} release was partially inhibited by xestospongin C and therefore one of the mechanism for H_2O_2-induced Ca^{2+} release from the agonist-sensitive stores might be interaction with the IP_3 receptors (Fig. 3A; $P < 0.05$, N = 14). In addition, Ca^{2+} release through the IP_3 receptors was not mediated by the stimulation of IP_3 synthesis induced by H_2O_2 since treatment of platelets for 2 hr with LiCl, to block the recycling of IP_3 [29], at concentrations that prevent thrombin-induced Ca^{2+} release (Fig. 3B, inset; N = 3), did not affect the response to H_2O_2. After incubation for 2 hr with LiCl 10 or 50 mM H_2O_2-induced Ca^{2+} release was 102.09 ± 7.75 and 97.21 ± 7.24% of control, respectively (also see Fig. 3B; N = 5–8). These results were confirmed using the phospholipase C inhibitor U-73122. Treatment of

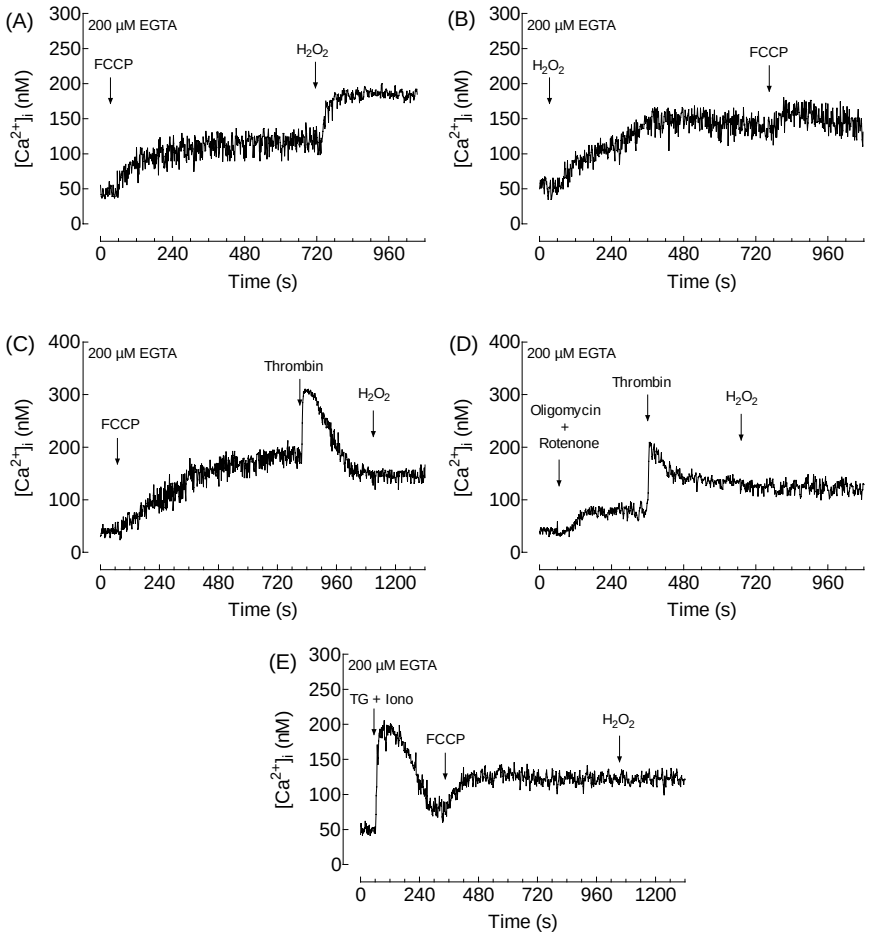

Fig. 2. Hydrogen peroxide releases Ca^{2+} from mitochondrial and agonist-sensitive Ca^{2+} stores in human platelets. (A) Fura-2-loaded human platelets were treated with 1 μM FCCP in a Ca^{2+}-free medium and 12 min later 1 mM H_2O_2 was added to the platelets suspension. (B) Cells were treated with 1 mM H_2O_2 and 12 min later 1 μM FCCP was added. (C) Human platelets were treated with 1 μM FCCP in a Ca^{2+}-free medium, 12 min later 1 U/mL thrombin was added to the platelets suspension and 1 mM H_2O_2 was added 5 min after thrombin. (D) Cells were treated with 10 μM oligomycin plus 10 μM rotenone, 5 min later 1 U/mL thrombin was added to the platelets suspension and 1 mM H_2O_2 was added 5 min after thrombin. (E) Cells were treated with 1 μM TG plus 50 nM ionomycin (Iono) and 5 min later 1 μM FCCP was added. H_2O_2 (1 mM) was added to the platelets suspension 12 min later. The traces shown are representative of six to seven separate experiments.

platelets for 10 min with 5 μM U-73122 did not significantly modify H_2O_2-induced Ca^{2+} release from agonist-sensitive stores (Fig. 3C; N = 4). To assess whether H_2O_2-stimulated Ca^{2+} release is dependent on the activity of the PI3-K cells were treated for 30 min with 100 nM wortmannin. As shown in Fig. 3C, H_2O_2 induced a similar Ca^{2+} release from agonist-sensitive stores in the absence or presence of wortmannin, suggesting that PI3-K activity is not require for this process.

Another possibility to explain the effect of H_2O_2 on the agonist-releasable stores consist of inhibition of SERCA, the ATPase that is reintroducing Ca^{2+} continuously into the stores. Using certain experimental conditions, similar to those previously applied to investigate the ability of PMCA to extrude Ca^{2+} [19] we have ascertained the activity of SERCA in human platelets. We have performed a series of experiments where $LaCl_3$ was present in the medium at a concentration of 1 mM, which effectively seals the cell, blocking both Ca^{2+} entry and extrusion [30]. As reported above, the buffer was supplemented with oligomycin plus rotenone to avoid Ca^{2+} release or uptake from mitochondria. Under these conditions, two mechanisms mediate Ca^{2+} mobilisation: Ca^{2+} release from agonist-sensitive stores, responsible for the rise in $[Ca^{2+}]_i$, and Ca^{2+} reuptake into the stores by SERCA, which returns $[Ca^{2+}]_i$ to basal levels. Therefore, the rate of decay of $[Ca^{2+}]_i$ to the basal concentration might be an indicative of SERCA activity.

Under our conditions, treatment of human platelets with thrombin (1 U/mL) induced a rapid and transient increase in $[Ca^{2+}]_i$, due to release of Ca^{2+} from agonist-sensitive stores, followed by reuptake into these stores by SERCA. When H_2O_2 was added simultaneously, thrombin induced a similar Ca^{2+} release, but the rate of decay of $[Ca^{2+}]_i$ to basal levels was significantly decreased (Fig. 4A). The decay constants were 0.0058 ± 0.0006 in H_2O_2-treated platelets and 0.0159 ± 0.0019 in paired controls ($P < 0.01$, N = 6). The inhibitory effect of H_2O_2 was confirmed by subsequent

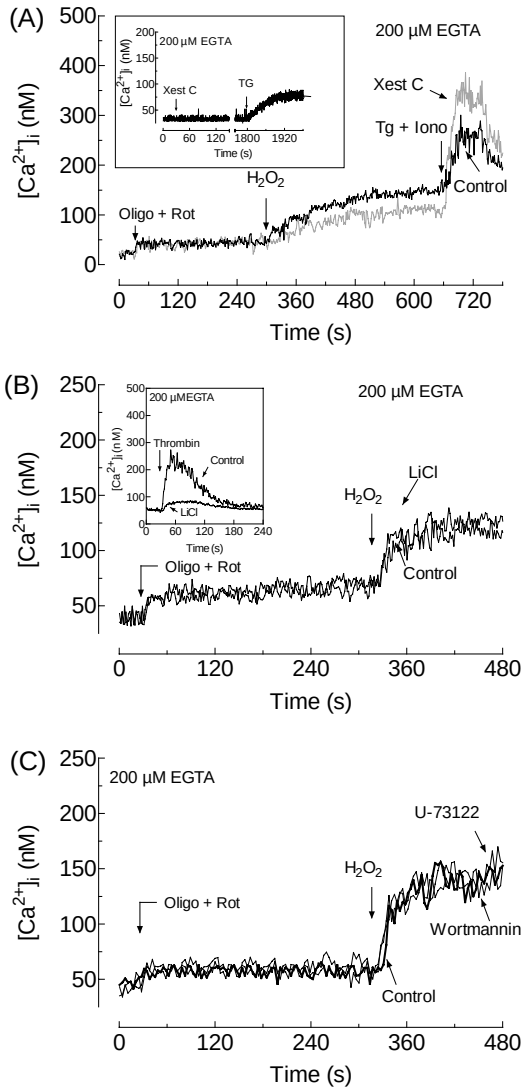

Fig. 3. Hydrogen peroxide-induced Ca^{2+} release from agonist-sensitive stores is reduced by xestospongin C but independent of IP_3 generation and phosphoinositide metabolism. (A) Fura-2-loaded human platelets were incubated in the absence or presence of 20 µM xestospongin C for 30 min at 37° as indicated and then treated in a Ca^{2+}-free medium with 10 µM oligomycin plus 10 µM rotenone, 5 min later 1 mM H_2O_2 was added to the platelets suspension and 1 µM TG plus 50 nM ionomycin (Iono) were added 12 min later to estimate the amount of Ca^{2+} remaining in the agonist-sensitive stores. *Inset*: Human platelets were incubated in the absence (solid line) or presence (dashed line) of 20 µM xestospongin C and 30 min later TG (200 nM) was added. (B) Human platelets were incubated in the absence or presence of 10 mM LiCl for 2 hr as indicated and then treated in a Ca^{2+}-free medium with 10 µM oligomycin plus 10 µM rotenone, 5 min later 1 mM H_2O_2 was added to the platelets suspension. Inset: cells were incubated in the absence or presence of 10 mM LiCl for 2 hr as indicated and then stimulated in a Ca^{2+}-free medium with 0.1 U/mL thrombin. (C) Cells were incubated at 37° in the presence of 5 µM U-73122 for 10 min, 100 nM wortmannin for 30 min or the vehicle (Control) as indicated and then treated in a Ca^{2+}-free medium with 10 µM oligomycin plus 10 µM rotenone, 5 min later 1 mM H_2O_2 was added to the platelet suspension. The traces shown are representative of 4–14 separate experiments.

testing of the Ca^{2+} remaining in the stores using TG plus ionomycin. In the presence of H_2O_2, Ca^{2+} remaining in the stores was found to be reduced by $39.3 \pm 10.7\%$ (Fig. 4; $P < 0.01$, $N = 6$).

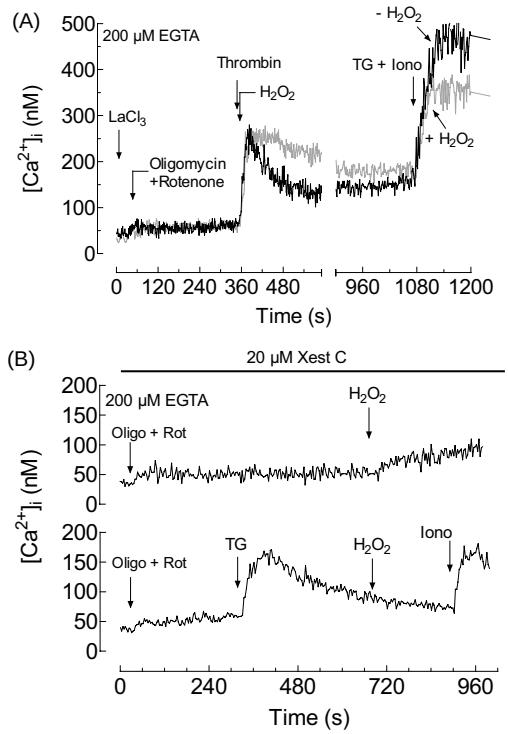

Fig. 4. Hydrogen peroxide-induced Ca^{2+} release from agonist-sensitive stores involves inhibition of SERCA. (A) Fura-2-loaded human platelets were incubated in a Ca^{2+}-free medium and in the presence of 1 mM $LaCl_3$ with 10 µM oligomycin plus 10 µM rotenone, 5 min later 1 U/mL thrombin and either 1 mM H_2O_2 ($+H_2O_2$) or the vehicle ($-H_2O_2$) were added to the platelets suspension as indicated and 1 µM TG plus 50 nM ionomycin (Iono) were added 12 min later to estimate the amount of Ca^{2+} remaining in the agonist-sensitive stores. (B) Fura-2-loaded human platelets were incubated with of 20 µM xestospongin C for 30 min at 37° as indicated and then treated in a Ca^{2+}-free medium with 10 µM oligomycin plus 10 µM rotenone. Cells were then treated with 1 µM TG (lower panel) or the vehicle (upper panel) and 6 min later 1 mM H_2O_2 was added to the platelets suspension followed by the addition of 500 nM ionomycin to estimate the amount of Ca^{2+} remaining in the agonist-sensitive stores. The traces shown are representative of three to six independent experiments.

Since there are still two possibilities to explain these differences: inhibition of SERCA or reduction of the IP_3 desensitisation after stimulation with thrombin, we further explored the involvement of SERCA in the effect of H_2O_2 using the protocol shown in Fig. 3A, with the addition of TG to inhibit SERCA prior the addition of H_2O_2. As shown in Fig. 4B, in cells pretreated with Xest C, inhibition of SERCA before the addition of H_2O_2 abolished H_2O_2-induced response although there still was Ca^{2+} remaining in the stores (as shown by the addition of ionomycin). These results indicate that the effect of H_2O_2 on Ca^{2+} release was mediated by IP_3 sensitisation and reduction of the activity of SERCA ($N = 3$).

Subsequently we investigate the effect of H_2O_2 on the ability of PMCA to extrude Ca^{2+} from the cytosol. To test this possibility we followed the protocol previously described by us [19]. As shown in Fig. 5A, cells were suspended in Ca^{2+}-free HBS supplemented with 10 µM rotenone plus 10 µM oligomycin to prevent H_2O_2-induced

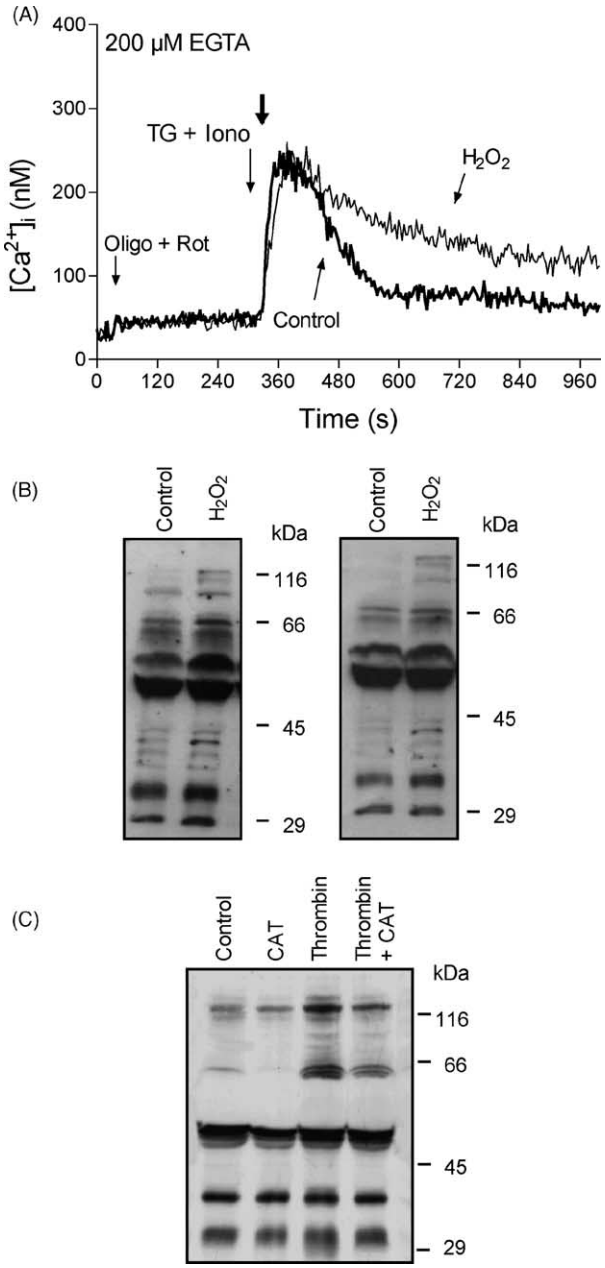

Fig. 5. Effect of H_2O_2 on restoration of $[Ca^{2+}]_i$ and tyrosine phosphorylation in human platelets. (A) Fura-2-loaded human platelets were resuspended in a Ca^{2+}-free medium supplemented with 10 μM oligomycin plus 10 μM rotenone and 5 min later 1 μM TG plus 50 nM ionomycin (Iono) and either 1 mM H_2O_2 (H_2O_2) or the vehicle (Control) were added to the platelets suspension as indicated by the arrow. Elevations in $[Ca^{2+}]_i$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_i$. Traces are representative of six independent experiments. (B) Control (left panel) and dimethyl BAPTA-loaded platelets (right panel) were treated in a Ca^{2+}-free medium (200 μM EGTA was added) with 1 mM H_2O_2, as indicated, and 12 min later samples were taken from the platelet suspension and lysed. (C) Human platelets were incubated for 10 min in the absence (Control) or presence of 300 U/mL catalase (CAT). Cells were stimulated with 0.5 U/mL thrombin, as indicated, and 5 min later samples were taken from the platelet suspension and lysed. Platelet proteins were analysed by SDS/10%-PAGE and subsequent Western blotting with a specific anti-phosphotyrosine antibody as described in the Section 2. Molecular masses (“kDa”) indicated on the right were determined using molecular-mass markers run in the same gel. The panels show results from one experiment representative of three to five others.

Ca^{2+} release from mitochondria and the intracellular Ca^{2+} stores were depleted by treatment with 1 μM TG plus 50 nM ionomycin. At the time of addition of the stimulus, 1 mM H_2O_2 or the vehicle were added. Using this protocol, again two mechanisms mediate Ca^{2+} mobilisation: Ca^{2+} release from agonist-releasable stores, responsible for the increase in $[Ca^{2+}]_i$, and Ca^{2+} efflux through the plasma membrane, which returns $[Ca^{2+}]_i$ to resting levels. We have previously demonstrated [19] that under our conditions the only mechanism involved in Ca^{2+} extrusion is the PMCA, therefore, the rate of decay of $[Ca^{2+}]_i$ to the basal concentration is an indicative of the ability of PMCA to extrude Ca^{2+} from the cell. As shown in Table 1, treatment of platelets with H_2O_2 almost completely inhibited the ability of PMCA to extrude Ca^{2+} ($P < 0.001$, N = 6). In addition, after treatment with H_2O_2, the return of $[Ca^{2+}]_i$ to basal was significantly reduced (5 min after treatment with TG + ionomycin the $[Ca^{2+}]_i$ was 153.7 ± 15.5 nM in H_2O_2-treated cells vs. 75.0 ± 15.9 nM in control), which might reflect the reduction in PMCA activity (Fig. 5A; $P < 0.01$, N = 6).

The effect of H_2O_2 was not prevented by previous addition of 2 mM DTT, a sulphydryl reducing agent, and thimerosal, a sulphydryl group oxidising agent, was unable to decrease the PMCA function suggesting that Ca^{2+} extrusion by the PMCA is insensitive to sulphydryl group oxidation in human platelets (Table 1; $P < 0.01$, N = 6).

Recently it has been shown that tyrosine phosphorylation of platelet PMCA leads to a substantial inhibition of its Ca^{2+}-ATPase activity [31]. To investigate whether tyrosine phosphorylation of PMCA might be the mechanism by which H_2O_2 induced inhibition of this pump, we examined the effect of H_2O_2 protein tyrosine phosphorylation in human platelets. As shown in Fig. 5B, treatment with 1 mM H_2O_2 increase the phosphotyrosine level either in normal platelets or in platelets loaded with dimethyl BAPTA to avoid increases in $[Ca^{2+}]_i$. These findings indicate that H_2O_2-induced protein tyrosine phosphorylation is independent of rises in $[Ca^{2+}]_i$.

Table 1
Effect of H_2O_2, thimerosal and DTT on PMCA activity in human platelets

Stimulatory agent	Decay constant	
	In the absence of DTT	In the presence of DTT (2 mM)
Vehicle	0.0017 ± 0.0001	0.0016 ± 0.0002
H_2O_2 (1 mM)	$0.0004 \pm 0.0001^{***}$	$0.0005 \pm 0.0001^{***}$
Thimerosal (5 μM)	0.0017 ± 0.0002	0.0016 ± 0.0003

Human platelets, suspended in a Ca^{2+}-free medium supplemented with rotenone (10 μM) plus oligomycin (10 μM), were stimulated with 1 μM TG plus 50 nM ionomycin to release Ca^{2+} from the intracellular stores. At the time of the addition of the stimulus, H_2O_2, thimerosal or the vehicle (as control) were added in the absence or presence of DTT (2 mM). The constant of the exponential decay of $[Ca^{2+}]_i$ to resting levels after the stimulation was calculated as described in Section 2. Values of the decay constants are presented as mean ± SEM, N = 6.

$^{***}P < 0.001$, compared to vehicle treatment.

In order to investigate whether H_2O_2-induced protein tyrosine phosphorylation is an event involved in cellular physiology we examined the effect of catalase on tyrosine phosphorylation induced by the physiological agonist thrombin. As shown in Fig. 5C, treatment of human platelets for 10 min with 300 U/mL catalase reduced thrombin-induced protein tyrosine phosphorylation by 40%, without having any effect on the phosphotyrosine content in resting conditions ($P < 0.05$, N = 5).

3.3. H_2O_2-induced release of Ca^{2+} from agonist-sensitive Ca^{2+} stores by oxidation of sulphydryl groups

It has been previously shown that the sulphydryl reagent thimerosal is able to sensitise the IP_3-induced Ca^{2+} release in platelets [15,32], an effect mediated by specific oxidation of sulphydryl groups. Consistent with this, treatment of human platelets with thimerosal (5 μM) in a Ca^{2+}-free medium resulted in a very slow and sustained increase in $[Ca^{2+}]_i$ (Fig. 6A; N = 10). Addition of thimerosal once the agonist-sensitive stores had been depleted by pretreatment with 1 μM TG plus 50 nM ionomycin was without response (Fig. 6B; N = 5), indicating that thimerosal released Ca^{2+} solely from the agonist-releasable pool.

As expected, the sustained increase in $[Ca^{2+}]_i$ induced by thimerosal was abolished by previous treatment of platelets with 2 mM DTT (Fig. 7A; N = 4). The effect of DTT on H_2O_2-induced Ca^{2+} release was also assessed. Under the same experimental conditions, Ca^{2+} release stimulated by 1 mM H_2O_2 was significantly reduced in the presence of 2 mM DTT although some Ca^{2+} release still remained (Fig. 7B; N = 12). To investigate the source of the DTT-independent H_2O_2-induced elevation in $[Ca^{2+}]_i$ we repeated these experiments in the presence of oligomycin and rotenone to deplete mitochondrial Ca^{2+} pools. As shown in Fig. 7C, under these conditions DTT was able to abolish the release of Ca^{2+} induced by H_2O_2 (N = 8). These findings indicate that oxidation of sulphydryl groups by H_2O_2 might be the mechanism involved in Ca^{2+} release from agonist-sensitive stores, while H_2O_2-induced Ca^{2+} release from mitochondrial pools seems to be mediated by a different process.

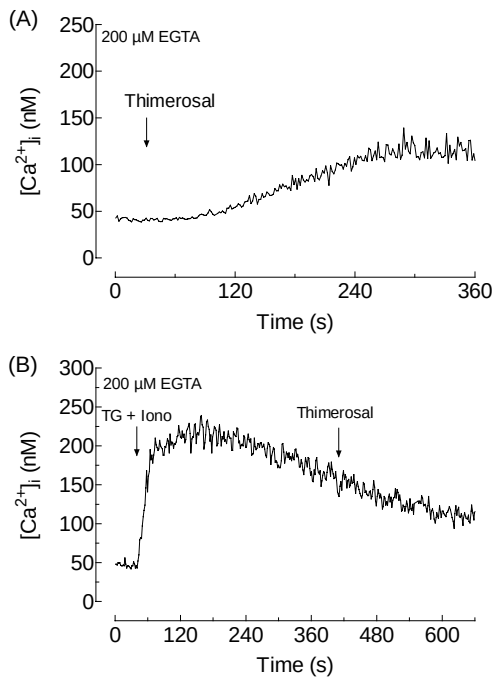

Fig. 6. Thimerosal releases Ca^{2+} from agonist-sensitive stores in human platelets. Fura-2-loaded human platelets were treated in a Ca^{2+}-free medium with 5 μM thimerosal (A) or with 1 μM TG plus 50 nM ionomycin (Iono) followed by the addition of 5 μM thimerosal 6 min later (B). The traces shown are representative of 5–10 experiments.

Table 2
Effect of thrombin and H_2O_2 on platelet aggregation

Time (min)	Aggregation (%)	
	Thrombin (0.5 U/mL)	H_2O_2 (1 mM)
0	0.0 ± 0.0	0.0 ± 0.0
0.5	4.8 ± 3.1	–
1	34.4 ± 15.8	16.6 ± 5.9
1.5	87.1 ± 19.0	–
2	92.17 ± 7.4	42.9 ± 16.4
3	–	50.0 ± 19.7
4	–	48.2 ± 18.9
5	–	51.6 ± 19.4
7	–	60.3 ± 16.2
10	–	81.6 ± 6.8

Platelet-rich plasma was exposed to thrombin (0.5 U/mL) or H_2O_2 (1 mM) for several times, as indicated at 37° under stirring conditions. Values are presented as percentage of aggregation and expressed as mean ± SEM, N = 6.

3.4. Effect of 1 mM H_2O_2 on platelet aggregation

In order to investigate the relevance of the cellular effects of H_2O_2 on platelet function we tested its effect on platelet aggregation. As shown in Table 2, addition of 1 mM H_2O_2 induced aggregation of stirred human platelets, although, in contrast with thrombin and other physiological agonists [22] that induce a more rapid aggregation of platelets, a considerable delay was observed between the time of addition of H_2O_2 and the initiation of aggregation (Table 2; N = 6).

4. Discussion

It has been shown that changes in the redox potential of sulphydryl groups plays an important role in Ca^{2+} homeostasis. Recent studies have reported that certain conditions that induce the opening of ryanodine receptors, such as micromolar Ca^{2+} concentrations, decrease its redox potential, which favours the oxidation of several sulphydryl groups residing on cysteine residues [18]. Alternatively, ryanodine receptors show an extraordinary sensitivity to reduction and oxidation of the hyperreactive

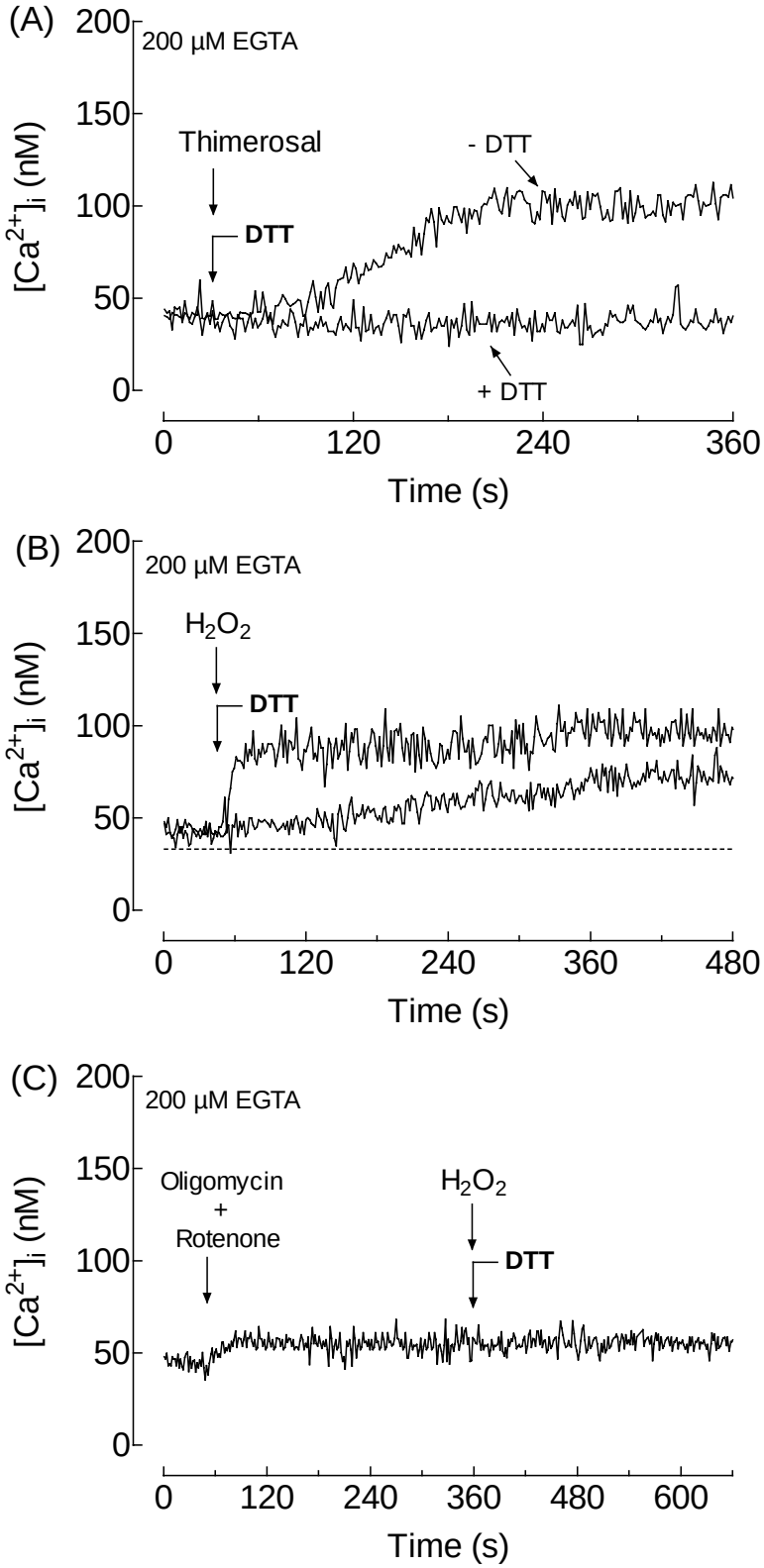

Fig. 7. Hydrogen peroxide-induced Ca^{2+} release from agonist-sensitive stores is mediated by oxidation of sulphydryl groups. Fura-2-loaded human platelets were incubated in a Ca^{2+}-free medium in the absence or presence of 2 mM DTT and then stimulated either with 5 µM thimerosal (A) or 1 mM H_2O_2 (B) or with 10 µM oligomycin plus 10 µM rotenone followed by the addition of 2 mM DTT and 1 mM H_2O_2 5 min later as indicated (C). The traces shown are representative of 4–12 independent experiments.

sulphydryl groups mentioned above [33,34]. The redox potential of the cytoplasm is −230 mV, as estimated in pancreatic cells [35]. Under these conditions ryanodine receptors are mostly inactivated by sulphydryl group reduction [18], but small changes in the redox potential mediated by oxidative stress, which leads the redox potential to become less negative, have been suggested to have a great influence on channel gating [18]. These findings suggest that specific sulphydryl groups play a relevant role in the modulation of channel gating at physiological or pathophysiological conditions. Since ryanodine and IP_3 receptors shares structural characteristics it might be expected that IP_3 receptors respond in a similar manner under changes in the redox potential.

Human platelets are a good model to investigate this event since ryanodine receptors expression have not been described as yet. The role of ROS, such as H_2O_2, in physiological platelet activation has been recently reported [8,12]. Human platelets generate ROS after activation by agonists such as collagen or thrombin, which regulates processes like the production of IP_3 or thromboxane B_2 [8,12].

Our results, in agreement with others [13,14], demonstrate that treatment of platelets with H_2O_2 induces Ca^{2+} release from agonist-sensitive Ca^{2+} stores. This effect might be mediated by sulphydryl group oxidation since thimerosal induced a similar response. In addition, H_2O_2 releases Ca^{2+} from the mitochondrial Ca^{2+} pool.

During the performance of the experiments cellular viability was tested using the tripan blue exclusion technique and retention of BCECF into the cells, two functional tests that indicate that treatment for 12 min with 1 mM H_2O_2 have negligible effects in cellular viability. In addition there are other data indicative of cell function after this treatment, such as Ca^{2+} mobilisation by agonists or lack of severe Fura-2 leakage monitored during the experiments.

In order to investigate the mechanism by which H_2O_2 induced Ca^{2+} release from agonist-sensitive stores we performed a series of experiments in the presence of rotenone, to block Ca^{2+} storage by mitochondria, and oligomycin, to avoid ATP depletion by the mitochondrial ATP synthase after mitochondrial uncoupling. To assess the role of the IP_3 receptors in H_2O_2-induced Ca^{2+} release we used the inhibitor of the IP_3 receptor function, xestospongin C. Pretreatment of platelets with xestospongin C at concentrations that inhibit thrombin-induced Ca^{2+} elevation [36] significantly reduced H_2O_2-induced release, suggesting that the IP_3 receptors are involved in the effect of H_2O_2. Ca^{2+} release induced by oxidants has been attributed to an increase in the affinity for IP_3 (see [37–39]); however, we have found that in the absence of IP_3 generation, by treatment with U-73122 or lithium at concentrations, at least as high as that shown by Berridge *et al.* [29], that inhibits thrombin-induced Ca^{2+} release, H_2O_2 was still able to release a similar extent of Ca^{2+} from agonist-sensitive stores. In addition, the effect of H_2O_2 does not depend on the PI3-K activity as demonstrated by the lack of effect of wortmannin in these cells. To our knowledge, these findings suggest for the first time that ROS releases Ca^{2+} from agonist-sensitive stores at least by a mechanism independent of IP_3 synthesis. In addition, these findings suggest that H_2O_2-evoked Ca^{2+} release is not mediated by IP_3 generation.

We have further investigated whether the SERCA might be a target of oxidative stress. To investigate this possibility we followed two different experimental procedures. One of the approaches is similar to the previously used by us to assess the ability of PMCA to extrude Ca^{2+} [19]. In this case, using certain protocols, as described in the Results section, we limited Ca^{2+} mobilisation to two mechanisms, Ca^{2+} release and reuptake by SERCA into the agonist-sensitive stores, responsible for the rise of $[Ca^{2+}]_i$ and return to basal levels, respectively. According to this, the rate of decay of $[Ca^{2+}]_i$ to basal values might be used as an indicative of the activity of SERCA. Additionally, we tested the ability of SERCA to store Ca^{2+} in the agonist-sensitive Ca^{2+} compartments. Our results indicate that H_2O_2 reduced both the rate of decay of $[Ca^{2+}]_i$ to basal and the amount of stored Ca^{2+}. Since these results might also been fully explained by IP_3 receptor sensitisation, described above, we alternatively explored the effect of platelet treatment with Xest C and inhibition of SERCA with TG before the addition of H_2O_2. Our observations, indicate that treatment of platelets with Xest C, which reduced the H_2O_2-induced response as shown in Fig. 3A, and TG, to inhibit SERCA, abolished the effect of H_2O_2, suggesting that Ca^{2+} release by H_2O_2 is mediated by both, IP_3 receptor sensitisation and inactivation of SERCA in human platelets.

As mentioned above, ryanodine receptors gating is sensitive to oxidation of sulphydryl groups. Hence, we have investigated whether this mechanism can explain the effect of H_2O_2 on Ca^{2+} release from intracellular stores. Consistent with previous studies using sulphydryl reagents [14,40–42], we have found that thimerosal, which at micromolar concentrations has been shown to sensitise the IP_3 receptor channel in platelets [15], is able to release Ca^{2+} from internal stores in human platelets. The effect of thimerosal was prevented by previous depletion of the agonist-sensitive stores, which indicates that thimerosal is unable to release Ca^{2+} from mitochondria and therefore ROS-induced Ca^{2+} efflux from mitochondria is mediated by other means different from oxidation of sulphydryl groups.

To confirm this possibility we used DTT a sulphydryl reducing agent that completely blocked the release of Ca^{2+} induced by thimerosal. Under our experimental conditions, DTT reduced H_2O_2-induced Ca^{2+} release; however, a significant amount of Ca^{2+} was still released by H_2O_2. To assess whether the source of the DTT-insensitive Ca^{2+} release is the mitochondrial store we depleted the mitochondrial Ca^{2+} pool using rotenone and oligomycin

previous treatment with DTT and H_2O_2. Mitochondrial uncoupling dissipates the membrane potential and leads to the release of accumulated Ca^{2+}; subsequent treatment with DDT abolished H_2O_2-induced Ca^{2+} release. These findings demonstrate that H_2O_2 induces Ca^{2+} release from agonist-sensitive Ca^{2+} stores by oxidation of sulphydryl groups. In contrast, H_2O_2 releases Ca^{2+} from the mitochondrial stores by a different mechanism. ROS, which may be produced in mitochondria by superoxide dismutase [43], might release Ca^{2+} from this organelle by activating the permeability transition pore, although the nature of this mechanism deserves further attention.

Finally, we have investigated the effect of H_2O_2 and thimerosal on the function of PMCA, the main responsible for Ca^{2+} extrusion in platelets at low $[Ca^{2+}]_i$ [19]. Our results, in agreement with previous studies [14,44], indicates that H_2O_2 strongly reduce the ability of PMCA to extrude Ca^{2+}. In addition, the $[Ca^{2+}]_i$ remained significantly elevated after stimulus in H_2O_2-treated cells. This difference might reflect the inhibitory role of H_2O_2 on the PMCA since the activity of SERCA is abolished by TG and we have previously shown that treatment with 1 μM TG plus 50 nM ionomycin completely deplete the intracellular Ca^{2+} stores in human platelets [45], and thus IP_3 receptor sensitisation is not expected to play any role.

The effect of H_2O_2 was unaffected by treatment with DTT. Consistent with this, thimerosal did not significantly modify the PMCA function, suggesting that sulphydryl groups oxidation is not responsible for this effect in human platelets. H_2O_2 induce a large number of biochemical modifications in the cell biology, such as an increase in the tyrosine phosphorylation state, which has been reported to inhibit the PMCA activity in human platelets [31]. The increase in the phosphotyrosine level induced by H_2O_2 was found to be independent on the rises in $[Ca^{2+}]_i$ and might be a mechanism by which H_2O_2 and physiological agonists modulate the PMCA activity.

Our findings in human platelets are similar to those reported in pancreatic acinar cells and pancreatic β cells [14,46], where H_2O_2 increased $[Ca^{2+}]_i$ by Ca^{2+} release from agonist-sensitive as well as mitochondrial stores and inhibition of the PMCA activity. In addition, the ability of H_2O_2 to inhibit the activity of SERCA has also been demonstrated in muscle cells, although this effect has been shown to be independent of sulphydryl group oxidation [16]. In contrast, in other cells, the involvement of mitochondria does not seem to be relevant [13,47,48] or it has not been further explored. These differences might be attributed to the idiosyncrasy of every cell type and also the different H_2O_2 concentrations used in these studies, which makes difficult to compare the results in different cellular models.

In conclusion our results provide evidence for the existence of a redox sensor in both the agonist-sensitive Ca^{2+} stores and mitochondria in human platelets. The redox sensor in the agonist-releasable pool might consist of hyperreactive sulphydryl groups present in the IP_3 receptors and SERCA. These groups are highly sensitive to oxidation by agonist-generated ROS or when platelets are exposed to oxidative stress under pathological situations. In addition, H_2O_2 reduces the ability of PMCA to extrude Ca^{2+} from the cytosol by a mechanism independent of sulphydryl group oxidation. All these modifications in the mechanisms involved in Ca^{2+} homeostasis result in the sustained increase in $[Ca^{2+}]_i$ induced by H_2O_2, which might be responsible, together with the stimulation of protein tyrosine phosphorylation, for the activation of platelet aggregation. Consistent with this, we have found a remarkable correlation between the slow pattern of Ca^{2+} mobilisation and aggregation by H_2O_2 compared to the rapid responses observed with agonist like thrombin. Considering the large number of cellular processes modulated by changes in $[Ca^{2+}]_i$, redox sensing might be of great importance in cellular physiology and may be involved in cellular alterations that occurs in ageing.

Acknowledgments

This work was supported by DGI (Spanish Ministry of Science and Technology) grant BFI2001-0624 and Consejería de Sanidad y Consumo (Junta de Extremadura) grant 02/0002. P.C.R. is supported by a DGI fellowship (BFI2001-0624). We thank Mercedes Gómez Blázquez for her technical assistance.

References

[1] Monteith GR, Roufogalis BD. The plasma membrane calcium pump: a physiological perspective on its regulation. Cell Calcium 1995;18: 459–70.
[2] Anzai K, Ogawa K, Kuniyasu A, Ozawa T, Yamamoto H, Nakayama H. Effects of hydroxyl radical and sulfhydryl reagents on the open probability of the purified cardiac ryanodine receptor channel incorporated into planar lipid bilayers. Biochem Biophys Res Commun 1998;249:938–42.
[3] Fowler CJ, Tiger G. The sulphydryl oxidizing reagent diamide affects phosphoinositide-mediated signal transduction: implications for the pathogenesis of Alzheimer's disease. Cell Signal 1998;10:399–406.
[4] Marengo JJ, Hidalgo C, Bull R. Sulfhydryl oxidation modifies the calcium dependence of ryanodine-sensitive calcium channels of excitable cells. Biophys J 1998;74:1263–77.
[5] Suzuki YJ, Ford GD. superoxide stimulates IP_3-induced Ca^{2+} release from vascular smooth muscle sarcoplasmic reticulum. Am J Physiol 1992;262:H114–6.
[6] Suzuki YJ, Forman HJ, Sevenian A. Oxidants as stimulators of signal transduction. Free Radic Biol Med 1997;22:269–85.
[7] Blockmans D, Deckmyn H, Vermylen J. Platelet activation. Blood Rev 1995;9:143–56.
[8] Wachowicz B, Olas B, Zbikowska HM, Buczynski A. Generation of reactive oxygen species in blood platelets. Platelets 2002;13:175–82.
[9] Iuliano L, Colavita AR, Leo R, Practico D, Violi F. Oxygen free radicals and platelet activation. Free Radic Biol Med 1997;22:999–1006.
[10] Seno T, Inoue N, Gao D, Okuda M, Sumi Y, Matsui K, Yamada S, Hirata K, Kawashima S, Tawa R, Imajoh-Ohmi S, Sakurai H,

Yokoyama M. Involvement of NADH/NADPH oxidase in human platelet ROS production. Thromb Res 2001;103:399–409.
[11] Maresca M, Colao C, Leoncini G. Generation of hydrogen peroxide in resting and activated platelets. Cell Biochem Funct 1992;10:79–85.
[12] Pignatelli P, Pulcinelli FM, Lenti L, Gazzaniga PP, Violi F. Hydrogen peroxide is involved in collagen-induced platelet activation. Blood 1998;91:484–90.
[13] Favero TG, Zable AC, Abramson JJ. Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. J Biol Chem 1995;270:25557–63.
[14] Pariente JA, Camello C, Camello PJ, Salido GM. Release of calcium from mitochondrial and nonmitochondrial intracellular stores in mouse pancreatic acinar cells by hydrogen peroxide. J Membr Biol 2001;179:27–35.
[15] Van Gorp RM, Feijge MA, Vuist WM, Rook MB, Heemskerk JW. Irregular spiking in free calcium concentration in single human platelets. Regulation by modulation of the inositol trisphosphate receptors. Eur J Biochem 2002;269:1543–52.
[16] Moreau VH, Castilho RF, Ferreira ST, Carvalho-Alves PC. Oxidative damage to sarcoplasmic reticulum Ca^{2+}-ATPase at submicromolar iron concentrations: evidence for metal-catalyzed oxidation. Free Radic Biol Med 1998;25:554–60.
[17] Keularts IM, Van Gorp RM, Feijge MA, Vuist WM, Heemskerk JW. Alpha(2A)-adrenergic receptor stimulation potentiates calcium release in platelets by modulating cAMP levels. J Biol Chem 2000; 275:1763–72.
[18] Xia R, Stangler T, Abramson JJ. Skeletal muscle ryanodine receptor is a redox sensor with a well defined redox potential that is sensitive to channel modulators. J Biol Chem 2000;275:36556–61.
[19] Rosado JA, Sage SO. Regulation of plasma membrane Ca^{2+}-ATPase by small GTPases and phosphoinositides in human platelets. J Biol Chem 2000;275:19529–35.
[20] Rosado JA, Jenner S, Sage SO. A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. J Biol Chem 2000; 275:7527–33.
[21] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[22] Irani K, Pham Y, Coleman LD, Roos C, Cooke GE, Miodovnik A, Karim N, Wilhide CC, Bray PF, Goldschmidt-Clermont PJ. Priming of platelet $\alpha_{IIb}\beta_3$ by oxidants is associated with tyrosine phosphorylation of β3. Arterioscler Thromb Vasc Biol 1998;18:1698–706.
[23] Thastrup O, Dawson AP, Scharff O, Foder B, Cullen PJ, Drobak BK, Bjerrum PJ, Christensen SB, Hamley MR. Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. Agents Actions 1989;27:17–23.
[24] Doni MG, Cavallini L, Alexandre A. Ca^{2+} influx in platelets: activation by thrombin and by the depletion of the stores. Effect of cyclic nucleotides. Biochem J 1994;303:599–605.
[25] Buckler KJ, Vaughan-Jones RD. Effects of mitochondrial uncouplers on intracellular calcium, pH and membrane potential in rat carotid body type I cells. J Physiol 1998;513:819–33.
[26] Cunningham ML, Soliman MS, Badr MZ, Matthews HB. Rotenone, an anticarcinogen, inhibits cellular proliferation but not peroxisome proliferation in mouse liver. Cancer Lett 1995;95:93–7.
[27] Wolvetang EJ, Johnson KL, Krauer K, Ralph SJ, Linnane AW. Mitochondrial respiratory chain inhibitors induce apoptosis. FEBS Lett 1994;339:40–4.
[28] Gafni J, Munsch JA, Lam TH, Catlin MC, Costa LG, Molinski TF, Pessah IN. Xestospongins: potent membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor. Neuron 1997;19:723–33.
[29] Berridge MJ, Downes CP, Hanley MR. Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. Biochem J 1982;206:587–95.
[30] Toescu EC, Petersen OH. Region-specific activity of the plasma membrane Ca^{2+} pump and delayed activation of Ca^{2+} entry characterize the polarized, agonist-evoked Ca^{2+} signals in exocrine cells. J Biol Chem 1995;270:8528–35.
[31] Dean WL, Chen D, Brandt PC, Vanaman TC. Regulation of the platelet plasma membrane Ca^{2+} ATPase by cAMP-dependent and tyrosine phosphorylation. J Biol Chem 1997;272:15113–9.
[32] Elferink JG. Thimerosal: a versatile sulphydryl reagent, calcium mobilizer, and cell function-modulating agent. Gen Pharmacol 1999;33:1–6.
[33] Missiaen L, Taylor CW, Berridge MJ. Spontaneous calcium release from inositol trisphosphate-sensitive calcium stores. Nature 1991; 352:241–4.
[34] Feng W, Allen PD, Pessah IN. Transmembrane redox sensor of ryanodine receptor complex. J Biol Chem 2000;275:35902–7.
[35] Hwang C, Sinskey AJ, Lodish HF. Oxidized redox state of glutathione in the endoplasmic reticulum. Science 1992;257:1496–502.
[36] Rosado JA, Sage SO. Coupling between inositol 1,4,5-trisphosphate receptors and human transient receptor potential channel 1 when intracellular Ca^{2+} stores are depleted. Biochem J 2000;350:631–5.
[37] Hilly M, Pietri-Rouxel F, Coquil JF, Guy M, Mauger JP. Thiol reagents increase the affinity of the inositol 1,4,5-trisphosphate receptor. J Biol Chem 1993;268:16488–94.
[38] Renard-Rooney DC, Joseph SK, Seitz MB, Thomas AP. Effect of oxidized glutathione and temperature on inositol 1,4,5-trisphosphate binding in permeabilized hepatocytes. Biochem J 1995;310:185–92.
[39] Vanlingen S, Sipma H, De Smet P, Callewaert G, Missiaen L, De Smedt H, Parys JB. Modulation of inositol 1,4,5-trisphosphate binding to the various inositol 1,4,5-trisphosphate receptor isoforms by thimerosal and cyclic ADP-ribose. Biochem Pharmacol 2001;61: 803–9.
[40] Adunyah SE, Dean WL. Effects of sulfhydryl reagents and other inhibitors on Ca^{2+} transport and inositol trisphosphate-induced Ca^{2+} release from human platelet membranes. J Biol Chem 1986;261: 13071–5.
[41] Van Gorp RMA, Van Dam-Mieras MCE, Hornstra G, Heemskerk JWM. Effect of membrane-permeable sulphydryl reagents and depletion of glutathione on calcium mobilisation in human platelets. Biochem Pharmacol 1997;53:1533–42.
[42] Mezna M, Michelangeli F. Effects of thimerosal on the transient kinetics of inositol 1,4,5-trisphosphate-induced Ca^{2+} release from cerebellar microsomes. Biochem J 1997;325:177–82.
[43] Richter C, Gogvadze V, Laffranchi R, Schlapbach R, Schweizer M, Suter M, Walter P, Yafee M. Oxidants in mitochondria: from physiology to diseases. Biochim Biophys Acta 1995;1271:67–74.
[44] Zaidi A, Michaelis ML. Effects of reactive oxygen species on brain synaptic plasma membrane Ca^{2+}-ATPase. Free Radic Biol Med 1999; 27:810–21.
[45] Rosado JA, Sage SO. Protein kinase C activates non-capacitative calcium entry in human platelets. J Physiol 2000;529:159–69.
[46] Krippeit-Drews P, Krämer C, Welker S, Lang F, Ammon HPT, Drews G. Interference of H_2O_2 with stimulus-secretion coupling in mouse pancreatic β-cells. J Physiol 1999;514:471–81.
[47] Roveri A, Coassin M, Maiorino M, Zamburlini A, Van Amsterdam FT, Ratti E, Ursini F. Effect of hydrogen peroxide on calcium homeostasis in smooth muscle cells. Arch Biochem Biophys 1992;297:265–70.
[48] Colston JT, Chandrasekar B, Freeman GL. A novel peroxide-induced calcium transient regulates interleukin-6 expression in cardiac-derived fibroblasts. J Biol Chem 2002;277:23477–83.