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Dual effect of hydrogen peroxide on store-mediated calcium entry in human platelets

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Abstract

Redox regulation is important for the modulation of cytosolic Ca^{2+} concentration. Hence, we have investigated the effect of H_2O_2 on store-mediated Ca^{2+} entry (SMCE). In fura-2-loaded human platelets treatment with H_2O_2 resulted in a concentration-dependent increase in Ca^{2+} release from intracellular stores, while the effect on Ca^{2+} entry was biphasic. In addition, 1 mM H_2O_2 reduced SMCE induced by agonists. The inhibitory effect of 1 mM H_2O_2 was prevented by inhibition of actin polymerization with cytochalasin D. Consistent with this, we found that 10 μM H_2O_2 and store depletion by treatment with thapsigargin plus ionomycin induced a similar temporal sequence of actin reorganization, while exposure to 1 mM H_2O_2 shifted the dynamics between polymerization and depolymerization in favor of the former. One millimolar H_2O_2-induced polymerization was reduced by treatment with methyl 2,5-dihydroxycinnamate and farnesylthioacetic acid, inhibitors of tyrosine kinases and Ras superfamily proteins, respectively. Finally, exposure to 1 mM H_2O_2 significantly increased store depletion-induced $p60^{src}$ activation. We conclude that H_2O_2 exerted a biphasic effect on SMCE. The inhibitory role of high H_2O_2 concentrations is mediated by an abnormal actin reorganization pattern involving both Ras- and tyrosine kinases-dependent pathways.

Keywords: Hydrogen peroxide; Actin cytoskeleton; Ca^{2+} entry; Tyrosine kinases; Ras; Human platelets

1. Introduction

Intracellular free calcium concentration ($[Ca^{2+}]_i$) is an important factor involved in the regulation of a large number of cellular functions. Platelets and other cells increase $[Ca^{2+}]_i$ by two mechanisms: Ca^{2+} release from intracellular stores and Ca^{2+} entry across the plasma membrane (PM), which is required for full platelet activation [1]. Non-excitable cells, such as platelets, lack voltage-activated Ca^{2+} channels, and the main mechanism for Ca^{2+} influx is store-mediated Ca^{2+} entry (SMCE), a process regulated by the degree of filling of the intracellular Ca^{2+} stores [2]. The mechanism of SMCE is still poorly understood and the models proposed to explain the communication between the intracellular Ca^{2+} stores and the PM can be divided into two classes: those suggesting a diffusible messenger and those suggesting a direct and physical coupling between elements in the two membranes (for review see [3]). Recently, the physical coupling model has received support from studies in different cell types, including platelets, which report that activation of SMCE shares properties with secretion [4–6], where the reorganization of the actin cytoskeleton appears to play a key role in the activation of SMCE. The actin cytoskeleton has been shown to play a dual role in the activation of SMCE: the filament network at the cell periphery acts as a negative clamp preventing constitutive coupling, on the other hand, actin polymerization is required apparently to mediate translocation of the endoplasmic reticulum (ER) to the PM to enable coupling to occur [4,6,7].

In platelets, the actin cytoskeleton is a very dynamic network organized in two major structures: a cytosolic actin filament network and a membrane-associated cytoskeleton [8]. Actin filament reorganization is modulated by several second messenger molecules, including proteins involved in intracellular signaling pathways, such as small

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Abbreviations: SMCE, store-mediated calcium entry; $[Ca^{2+}]_i$, intracellular free calcium concentration; PM, plasma membrane; ER, endoplasmic reticulum; PBS, phosphate-buffered saline; HBS, HEPES-buffered saline; DTT, dithiothreitol; M-2,5-DHC, methyl 2,5-dihydroxycinnamate; FTA, farnesylthioacetic acid; Cyt D, cytochalasin D; TG, thapsigargin; Iono, ionomycin; PMCA, plasma membrane Ca^{2+} ATPase.

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GTPases of the Ras superfamily [9–11] and tyrosine kinases either inducing actin polymerization [12] or membrane association [13]. The actin cytoskeleton is also the target of several agents such as reactive oxygen species (ROS), although the mechanism by which the structure and spatial organization is altered by these oxidants remains unclear. In several cell types, oxidative stress induces alterations such as dissociation of actin filaments from α-actinin, which associates the cytoskeleton with actin-binding proteins, shortening of filaments with the aggregation into short bundles and alteration in the polymerization–depolymerization equilibrium that results in an increase in the actin filament content with an abnormal cellular distribution [14,15]. These alterations in the organization of the actin cytoskeleton by ROS have been attributed to primary events, such as oxidation of sulfydryl groups present in actin and other actin-associated proteins, or second messenger-mediated mechanisms, including, changes in $[Ca^{2+}]_i$ or the concentration of cyclic nucleotides and the activation of protein kinases (for review see [14]).

Human platelets are exposed to ROS present in blood and also they have been shown to generate ROS. Micromolar levels of H_2O_2 have been reported in human plasma and whole blood and even higher levels under certain physiological conditions, such as exercise [16,17]. In addition, under pathological situations, such as diabetes or ischemia/reperfusion injury the level of H_2O_2 might locally increase [18,19]. Furthermore, human platelets generate and release ROS under physiological stimulation [20,21]. Platelet stimulation with collagen 10 or 50 μg/mL induced H_2O_2 production which reached a concentration close to 1 mM [20]. SMCE has been reported to be reduced by treatment with ROS, a mechanism mediated by the activation of PKC leading to membrane depolarization and increased Ca^{2+} extrusion [22]. We report here that H_2O_2 has both positive and negative effects on SMCE depending on the concentration. The inhibitory actions of high H_2O_2 concentrations are due to an altered temporal pattern of actin cytoskeleton remodeling involving the activation of tyrosine kinases and Ras superfamily proteins in human platelets.

2. Materials and methods

2.1. Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes. Apyrase (grade VII), aspirin, bovine serum albumin, paraformaldehyde, Nonidet P-40, fluorescein isothiocyanate-labeled phalloidin, thrombin, hydrogen peroxide (H_2O_2), thimerosal, dithiotreitol (DTT), dimethyl BAPTA and thapsigargin (TG) were from Sigma. Methyl 2,5-dihydroxycinnamate (M-2,5-DHC), cytochalasin D (Cyt D), rotenone, oligomycin and ionomycin (Iono) were from Calbiochem. Farnesylthioacetic acid (FTA) was from Alexis Corporation. Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology. Horseradish peroxidase-conjugated ovine anti-mouse IgG antibody (NA931) was from Amersham. Anti-phospho-*c*-Src (Y-416) antibody and horseradish peroxidase-conjugated goat anti-rabbit IgG antibody were from Santa Cruz. Enhanced chemiluminescence detection reagents were from Pierce. All other reagents were purchased from Panreac.

2.2. Platelet preparation and measurement of intracellular free calcium concentration ($[Ca^{2+}]_i$)

Fura-2-loaded platelets were prepared as described previously [11]. Briefly, 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 HEPES-buffered saline (HBS) containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, pH 7.45 and supplemented with 0.1% w/v bovine serum albumin and 40 μg/mL apyrase. When the experiments were performed in a Ca^{2+}-free medium EGTA (200 μM) was added. Fluorescence was recorded from 2 mL aliquots of magnetically stirred platelet suspension (10^8 cells/mL) at 37° using a Fluorescence Spectrophotometer (Varian Ltd.) with excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in $[Ca^{2+}]_i$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz *et al*. [23].

Ca^{2+} release and entry were estimated using the integral against time of the rise in $[Ca^{2+}]_i$ above basal level (the level before the addition of the Ca^{2+}-releasing agent or $CaCl_2$) for 12 or 2.30 min, respectively, after the addition of the Ca^{2+}-mobilizing stimulus or $CaCl_2$ and expressed as nM [11,24]. Ca^{2+} entry was corrected by subtraction of the increase in $[Ca^{2+}]_i$ due to leakage of the indicator.

Cell viability was assessed using calcein and trypan blue. For calcein loading, cells were incubated for 30 min with 5 μM calcein-AM at 37°, centrifuged and the pellet was resuspended in fresh HBS. Fluorescence was recorded from 2 mL aliquots using a Shimadzu Spectrophotometer. Samples were excited at 494 nm and the resulting fluorescence was measured at 535 nm. After treatment with 10 μM or 1 mM H_2O_2 cells were centrifuged and resuspended in fresh HBS. The calcein fluorescence remaining in the cells after treatment with 10 μM H_2O_2 was the same as in control, and slightly smaller after treatment with 1 mM H_2O_2, suggesting that under our conditions there was no cellular damage. The results obtained with calcein were confirmed using the trypan blue exclusion technique. Ninety-five percent of cells were viable after treatment with 10 μM H_2O_2, similar to that observed in our resting platelet suspensions, and 92% after treatment with 1 mM H_2O_2, at least during the performance of the experiments.

2.3. Measurement of F-actin content

The F-actin content of resting and activated platelets was determined according to a previously published procedure [11]. Briefly, washed platelets (2×10^8 cells/mL) were activated in HBS. Samples of platelet suspension (200 μL) were transferred to 200 μL ice-cold 3% (w/v) formaldehyde in phosphate-buffered saline (PBS) for 10 min. Fixed platelets were permeabilized by incubation for 10 min with 0.025% (v/v) Nonidet P-40 detergent dissolved in PBS. Platelets were then incubated for 30 min with fluorescein isothiocyanate-labeled phalloidin (1 μM) in PBS supplemented with 0.5% (w/v) bovine serum albumin. After incubation the platelets were collected by centrifugation for 60 s at 7200 *g* and resuspended in PBS. Staining of 2×10^7 cells/mL was measured using a Varian Fluorescence Spectrophotometer. Samples were excited at 496 nm and emission was at 516 nm.

2.4. Protein tyrosine phosphorylation

Protein tyrosine phosphorylation was detected by gel electrophoresis and Western blotting [11]. Platelets stimulation (2×10^8 cells/mL) was terminated by mixing with an equal volume of 2× Laemmli's buffer [25] with 10% dithiothreitol (DTT) followed by heating for 5 min at 95°. One-dimensional SDS–electrophoresis was performed with 10% polyacrylamide minigels (50 μg total protein loaded/sample) 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. p60^{src} activation

Autophosphorylation of p60^{src} at Tyr-416 and thus activation was detected by gel electrophoresis and Western blotting as described above. Immunodetection of p60^{src} phosphorylated at Tyr-416 was achieved using the anti-phospho-*c*-Src (Y-416) antibody, diluted 1:1000, according to manufacturer's instruction, in TBST for 2 hr [26]. 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 goat anti-rabbit IgG antibody diluted 1:10,000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were exposed to photographic films and the optical density was estimated using scanning densitometry.

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. Dose-dependent effect of H_2O_2 on Ca^{2+} release and SMCE in human platelets

H_2O_2 has been reported to release Ca^{2+} from agonist-sensitive stores and mitochondrial pools [27]. Since the aim of the present study is to investigate the effect of H_2O_2 on SMCE, and to avoid interference with mitochondrial Ca^{2+} release, 10 μM rotenone, an inhibitor of complex I of the respiratory chain that dissipates the membrane potential [28], plus oligomycin, to prevent ATP depletion after mitochondrial membrane potential collapse [29], were added to the buffer to deplete the mitochondrial pool. We found that addition of rotenone plus oligomycin to the platelets suspension induced a small and sustained increase in $[Ca^{2+}]_i$ but did not induce SMCE (data not shown). In the absence of extracellular Ca^{2+}, treatment of human platelets with increasing concentrations of H_2O_2 (10 μM–5 mM) resulted in a concentration-dependent increase in Ca^{2+} release from the intracellular stores (Fig. 1A (solid circles) and 1B, N = 6). However, treatment with H_2O_2 induced a concentration-dependent and biphasic increase in Ca^{2+} entry in human platelets (Fig. 1A (open circles) and 1B, N = 6). H_2O_2 induced a detectable Ca^{2+} entry at 10 μM and a maximal effect at 100 μM. At these two concentrations there was a positive correlation between Ca^{2+} release and entry, in contrast, the ability of H_2O_2 to induce Ca^{2+} entry decreased at higher H_2O_2 concentrations despite the greater effect on Ca^{2+} release (Fig. 1).

3.2. Effect of H_2O_2 on store depletion- and agonist-induced SMCE in human platelets

In a Ca^{2+}-free medium supplemented with rotenone and oligomycin, treatment of platelets with 1 μM TG, a specific inhibitor of the Ca^{2+}-ATPase of internal stores [27] plus a low concentration of Iono (50 nM, required for extensive

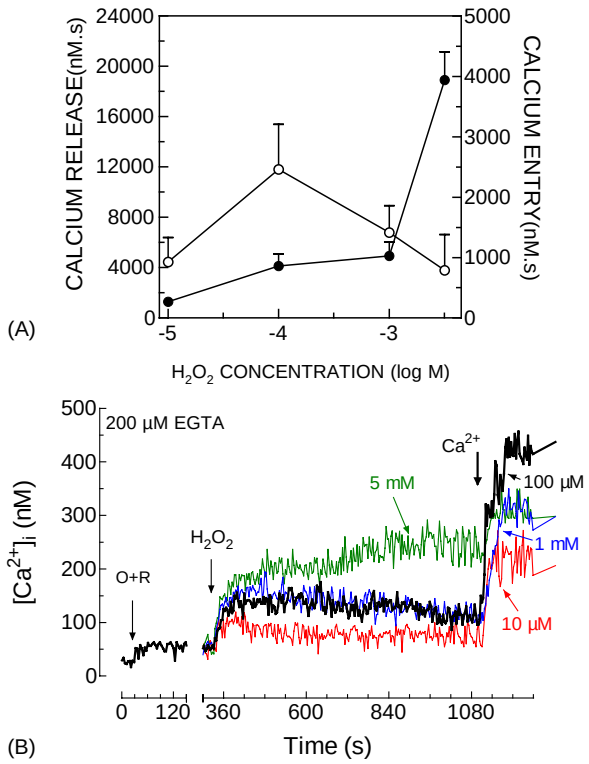

Fig. 1. Concentration dependence of H_2O_2-induced SMCE. (A) Fura-2-loaded human platelets were treated with various concentrations of H_2O_2 (10 µM–5 mM) in a Ca^{2+}-free medium (200 µM EGTA was added) and 12 min later $CaCl_2$ (final concentration 1 mM) was added to the medium to initiate Ca^{2+} entry. H_2O_2-induced Ca^{2+} release (●) and entry (○) were calculated as described in Section 2 and are expressed as means ± SEM for six separate experiments. (B) Fura-2-loaded human platelets were suspended in a Ca^{2+}-free medium (200 µM EGTA added) as described in Section 2. Cells were then treated with increasing concentrations of H_2O_2, as indicated, and 12 min later $CaCl_2$ (final concentration 1 mM) was added to the medium to initiate Ca^{2+} entry. Traces are representative of six independent experiments.

depletion of the agonist-sensitive Ca^{2+} stores in platelets where two stores, with high and low Ca^{2+} leakage rates, have been reported) [28] resulted in a prolonged increase in $[Ca^{2+}]_i$ due to the release of Ca^{2+} from intracellular pools. The subsequent addition of Ca^{2+} (1 mM) to the external medium induced a sustained elevation in $[Ca^{2+}]_i$ indicative of SMCE (Fig. 2). Platelet treatment with H_2O_2 (10 µM) at the time of TG + Iono did not modify either Ca^{2+} release or entry patterns (Fig. 2A). In contrast, addition of 1 mM H_2O_2 at the time of TG + Iono modified the Ca^{2+} release pattern, so that $[Ca^{2+}]_i$ decay was slowed. In addition, 1 mM H_2O_2 significantly attenuated TG + Iono-induced SMCE by 63% (Fig. 2B, $P < 0.01$, N = 5). Similar results were obtained when store depletion was achieved using the physiological agonist thrombin (1 U/mL). In the presence of 1 mM H_2O_2 thrombin-induced SMCE was reduced by 40% (Fig. 2C, $P < 0.05$, N = 5). When the experiments were performed in a buffer without rotenone and oligomycin the results obtained were very similar. Addition of H_2O_2 (1 mM) to the platelets suspension reduced SMCE-evoked by TG + Iono or thrombin by 65 and 43%, respectively (Fig. 2D and E, $P < 0.05$, N = 5–7).

To further explore the effect of 1 mM H_2O_2 on SMCE, we tested its effect on the maintenance of this process. In a buffer supplemented with rotenone plus oligomycin, addition of H_2O_2 once the agonist-sensitive Ca^{2+} stores had been depleted by TG + Iono or thrombin reduced SMCE by 25 and 15%, respectively (Fig. 2F and G, $P < 0.05$, N = 5). The smaller effect of H_2O_2 on the activation and maintenance of SMCE support previous findings suggesting that both phases of Ca^{2+} entry are mediated by different intracellular pathways [12,26].

To investigate whether the effect of 1 mM H_2O_2 might be mediated by sulfydryl group oxidation, we tested the effect of the sulfydryl reducing agent DTT on H_2O_2-induced reduction of SMCE. As shown in Fig. 3, treatment of human platelets with 2 mM DTT did not modify either Ca^{2+} release or SMCE stimulated by TG + Iono (Fig. 3A, N = 8). In addition, DTT prevented the effect of treatment with 1 mM H_2O_2 both on Ca^{2+} release and entry induced by TG + Iono (Fig. 3B, N = 8), suggesting that the inhibitory effect of 1 mM H_2O_2 on SMCE is mediated by oxidation of sulfydryl groups.

3.3. *Cytochalasin D prevents the inhibitory effect of H_2O_2 on SMCE in human platelets*

Recent studies by us have reported that the actin cytoskeleton plays both facilitating and inhibitory roles in the activation of SMCE by a secretion-like coupling between the IP_3 receptor type II and hTrp1 in human platelets [6,7]. The actin cytoskeleton is one of the targets of oxidative stress, which modifies its structure and spatial organization. Hence we have studied the involvement of the actin network in the inhibitory effect of high H_2O_2 concentrations on SMCE in platelets. In a previous report [11], we have demonstrated that inhibition of actin polymerization by treatment with 10 µM Cyt D induced a time-dependent and biphasic effect of SMCE in these cells. Cyt D slightly increased SMCE after 1-min treatment, a process mediated by erosion of the actin clamp that prevent constitutive coupling in these cells [7], and inhibition after 20-min treatment due to impairment of the actin polymerization-mediated trafficking of ER portions towards the PM. In an equilibrium between both phenomena 10-min treatment with Cyt D did not modify SMCE although a clear reduction in TG-induced actin polymerization was observed [11].

As shown in Table 1, treatment of platelets with 1 mM H_2O_2 resulted in a significant increase in the actin filament content and increased the polymerizing effects of TG + Iono and the physiological agonist thrombin. Treatment of human platelets with 10 µM Cyt D for 10 min did not alter the actin filament content of resting cells, confirming that treadmilling in platelets is slow [30], but abolished actin polymerization induced by 1 mM H_2O_2

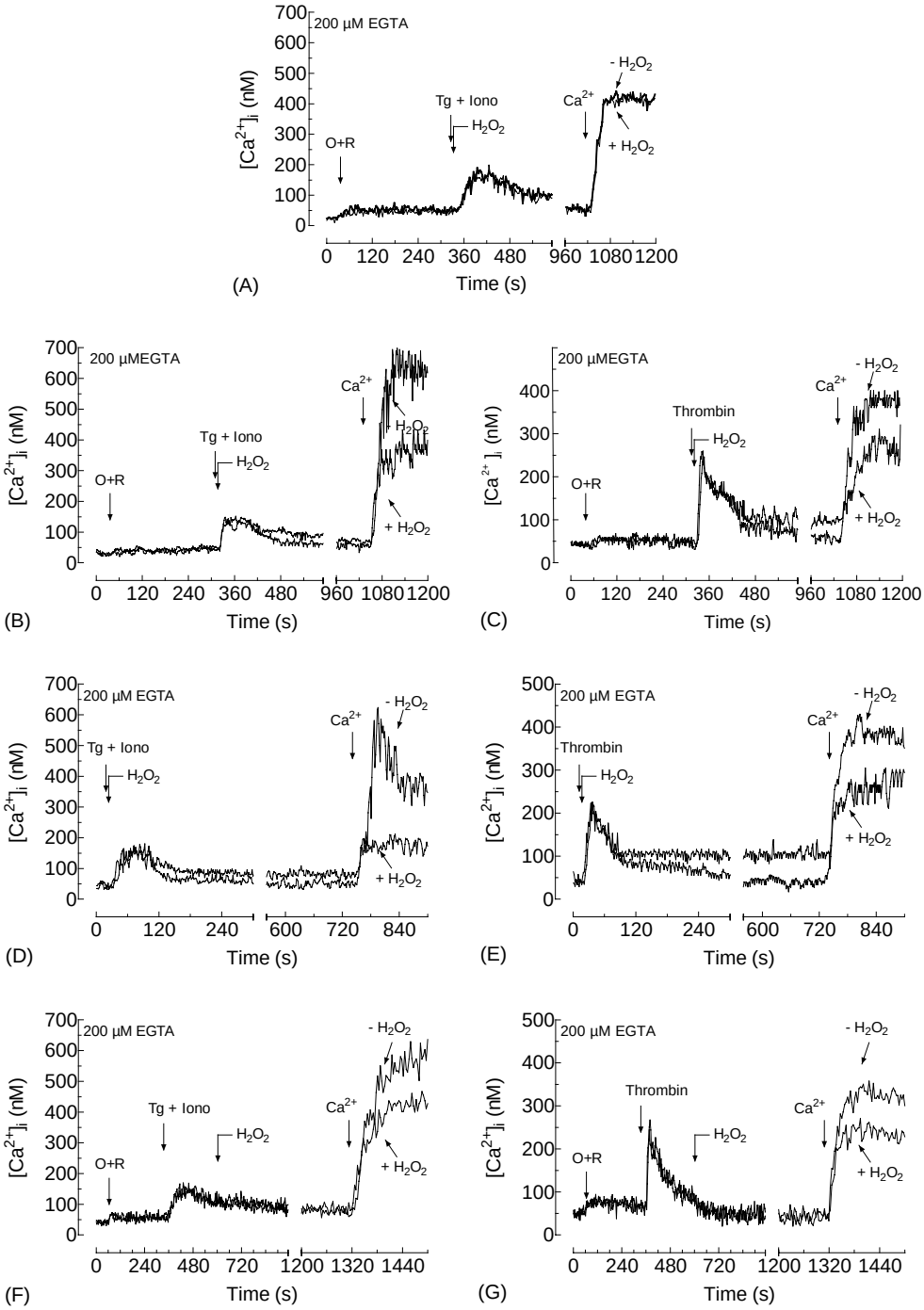

Fig. 2. Effect of H_2O_2 on store depletion- and agonist-induced Ca^{2+} entry in human platelets. (A–C) Human platelets were treated in a Ca^{2+}-free medium with 10 µM oligomycin plus 10 µM rotenone and 5 min later stimulated with either 1 µM TG + 50 nM Iono (A and B) or 1 U/mL thrombin (C) in the absence or presence of 10 µM (A) or 1 mM (B and C) H_2O_2, as indicated, and 12 min later $CaCl_2$ (1 mM) was added to the medium to initiate Ca^{2+} entry. (D and E) Fura-2-loaded human platelets were treated in a Ca^{2+}-free medium (200 µM EGTA was added) with 1 µM TG + 50 nM Iono (D) or 1 U/mL thrombin (E) in the absence or presence of 1 mM H_2O_2, as indicated, and 12 min later $CaCl_2$ (final concentration 1 mM) was added to the medium to initiate Ca^{2+} entry. (F and G) Human platelets were treated in a Ca^{2+}-free medium with 10 µM oligomycin plus 10 µM rotenone and then stimulated with either 1 µM TG + 50 nM Iono (F) or 1 U/mL thrombin (G). Four minutes later 1 mM H_2O_2 or the vehicle was added and 12 min later $CaCl_2$ (1 mM) was added to the medium. Changes in $[Ca^{2+}]_i$ were monitored as described in Section 2. Traces are representative of five to seven independent experiments.

(Table 1). In addition, Cyt D treatment reduced actin polymerization induced by TG + Iono and thrombin and abolished the increasing effect of H_2O_2 on the agonists-induced polymerization (Table 1). Next we tested the effect of Cyt D on the inhibitory role of 1 mM H_2O_2 in SMCE. As shown in Fig. 4, treatment of platelets for 10 min with 10 µM Cyt D had no effect *per se* on Ca^{2+} release stimulated by TG + Iono (Fig. 4A and B). As previously reported [11] treatment for 10 min with Cyt D did not alter SMCE stimulated by TG + Iono. Interestingly, Cyt D

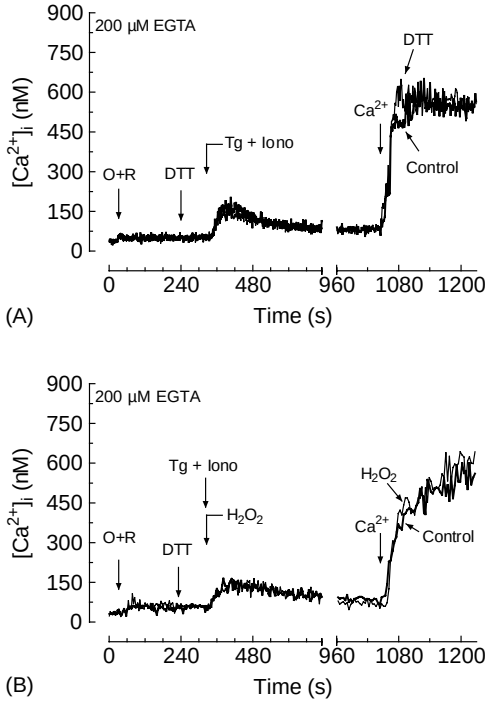

Fig. 3. DTT impairs H_2O_2-induced reduction in SMCE. Fura-2-loaded human platelets were treated in a Ca^{2+}-free medium (200 μM EGTA added) with 10 μM oligomycin plus 10 μM rotenone. Five minutes later DTT (2 mM) was added and then cells were stimulated with 1 μM TG + 50 nM Iono in the absence (A) and presence of 1 mM H_2O_2 (B). $CaCl_2$ (final concentration 1 mM) was added to the medium 12 min later to initiate Ca^{2+} entry. Changes in $[Ca^{2+}]_i$ were monitored as described in Section 2. Traces shown are representative of eight independent experiments.

prevented the inhibition observed in TG + Iono-induced SMCE after treatment with 1 mM H_2O_2, suggesting that these effects might be mediated by actin polymerization (Fig. 4A and C).

Table 1
Effect of H_2O_2 on the F-actin content in resting and store depleted human platelets

Stimulatory agent	Filamentous actin (% of control)	
	Control	Cytochalasin D
None	100.0 ± 0.0	99.5 ± 7.7
H_2O_2	113.8 ± 4.3*	97.3 ± 7.2§
TG + Iono	119.1 ± 4.1	108.7 ± 6.2
TG + Iono + H_2O_2	137.1 ± 13.1*	107.8 ± 5.7§
Thrombin	122.6 ± 13.8	112.3 ± 14.3
Thrombin + H_2O_2	144.7 ± 12.6	115.9 ± 12.2

Human platelets were suspended in a Ca^{2+}-free medium as described in Section 2. Cells were preincubated for 10 min with 10 μM Cyt D or the vehicle (control) and then stimulated with either 1 μM TG plus 50 nM Iono or 1 U/mL thrombin in the absence or presence of 1 mM H_2O_2. Samples were removed 5 s before and 12 min after the addition of the stimulus and F-actin content was determined as described in Section 2. Values given are the F-actin content expressed as a percentage of control and are presented as mean ± SEM of 8 to 17 separate determinations.

* $P < 0.05$ compared with the actin filament content in the absence of H_2O_2.

§ $P < 0.05$ compared with the actin filament content in the absence of Cyt D.

In view of the results we repeated the experiments shown in Fig. 1A in platelets treated for 10 min with 10 μM Cyt D and the results are depicted in Fig. 4D. Consistent with the results presented above, in Cyt D-treated platelets H_2O_2 induced a concentration-dependent increase both in Ca^{2+} release and entry, preventing the inhibition of SMCE by high concentrations of H_2O_2.

3.4. Temporal sequence of actin polymerization induced by H_2O_2 and TG + Iono

The secretion-like coupling has been proposed as the most likely model of SMCE in human platelets and other non-excitable cells [4–6,26]. According to this model, as for secretion, agonists should reorganize the actin microfilaments in order to dissipate the actin cortical layer, which acts as a negative clamp, and provide a support for the coupling. Physiological secretagogues induce an initial net depolymerization, that lasts for a few minutes, followed by an increase in the actin filament content, which leads to secretion [15,31]. Consistent with this, treatment of human platelets with 10 μM H_2O_2 (Fig. 5A) or 1 μM TG + 50 nM Iono (Fig. 5B) induced a rapid decrease in the actin filament content, which was maximal after 30 s of stimulation and by 4 min returned to prestimulation levels to increase for at least 12 min. On the other hand, treatment of platelets with 1 mM H_2O_2 alone hardly induced net polymerization and clearly inhibited the initial depolymerization induced by TG + Iono when added together. As shown in Fig. 5, treatment of platelets with 1 mM H_2O_2 in combination with TG + Iono induced a rapid actin polymerization in platelets that was higher than that induced by TG + Iono alone. These differences in the reorganization pattern of the actin network might be responsible of the inhibition of SMCE.

3.5. Involvement of tyrosine kinases and Ras superfamily proteins in actin polymerization induced by 1 mM H_2O_2

Previous studies have reported that tyrosine kinase activity is required for SMCE in several cell types [32,33], including human platelets [34], where tyrosine phosphorylation is essential for actin polymerization induced by store depletion [12]. In a Ca^{2+}-free medium human platelets were treated for 12 min with 1 μM TG + 50 nM Iono, to induce extensive depletion of agonist-sensitive stores, and 12 min later $CaCl_2$ (1 mM) was added. Samples were taken from the platelet suspension at 30 s before and 12 min after the addition of TG + Iono and 3 min after $CaCl_2$ addition. Our results indicate that treatment of platelets with TG + Iono resulted in a significant increase in the phosphotyrosine level. Interestingly, the phosphotyrosine level was significantly increased when TG + Iono were added in combination with 1 mM H_2O_2. Similar effects were observed when the experiments were

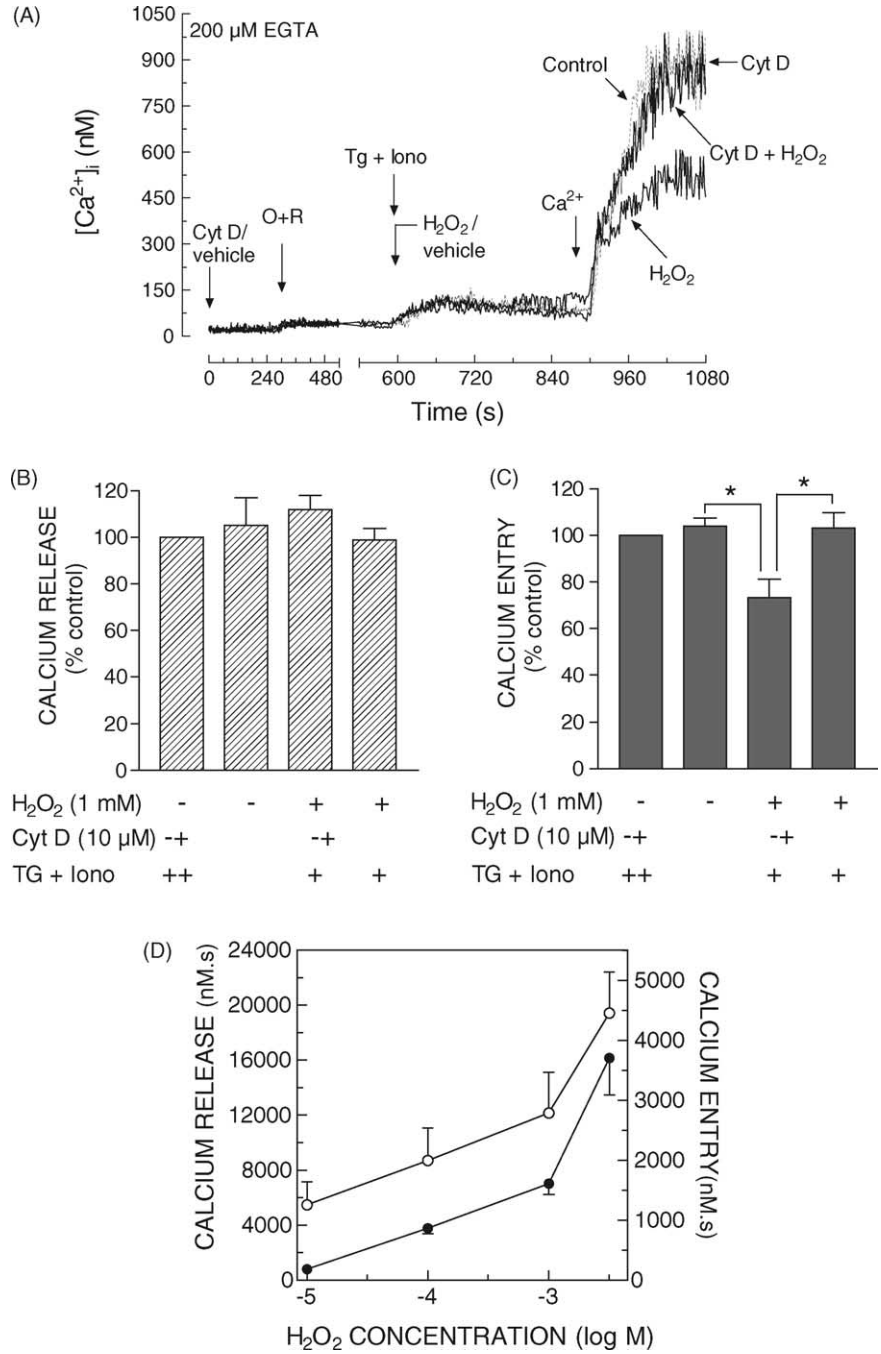

Fig. 4. Cytochalasin D prevents H_2O_2-induced reduction of SMCE. (A) Fura-2-loaded human platelets were treated with 10 μM Cyt D in a Ca^{2+}-free medium (200 μM EGTA added), after 5 min 10 μM oligomycin plus 10 μM rotenone were added and 5 min later cells were stimulated with 1 μM TG + 50 nM Iono in the absence and presence of 1 mM H_2O_2. $CaCl_2$ (1 mM) was added to the medium 12 min later to initiate Ca^{2+} entry. (B and C) Histograms indicating the percentage of Ca^{2+} release (B) or entry (C) under the above conditions relative to their control (vehicle was added). Ca^{2+} release and entry were determined as described in Section 2. Values are means ± SEM for 9 to 12 experiments. $^*P < 0.05$. (D) Platelets were treated with various concentrations of H_2O_2 (10 μM–5 mM) in a Ca^{2+}-free medium (200 μM EGTA was added) and 12 min later $CaCl_2$ (final concentration 1 mM) was added to the medium to initiate Ca^{2+} entry. H_2O_2-induced Ca^{2+} release (●) and entry (○) were calculated as described in Section 2 and are expressed as means ± SEM for six separate experiments.

performed in platelets heavily loaded with the Ca^{2+} chelator dimethyl BAPTA, used for this study so as to eliminate Ca^{2+}-evoked but not store depletion-evoked tyrosine phosphorylation [12] (Fig. 6 and Table 2, $P < 0.05$, $N = 6$). BAPTA loading prevented Ca^{2+} mobilization induced by TG + Iono either in the absence or presence of 1 mM H_2O_2 (data not shown). To test whether the effect of 1 mM H_2O_2 on actin polymerization is mediated by tyrosine kinases we have examined the effect of M-2,5-DHC, a specific tyrosine kinase inhibitor [12]. Platelets

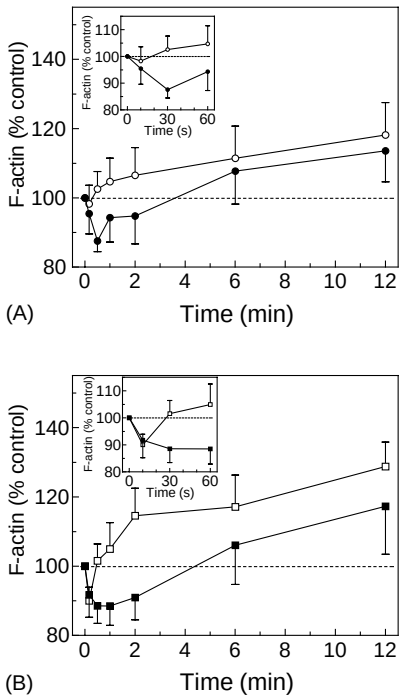

Fig. 5. Time course of actin polymerization induced by hydrogen peroxide and TG + Iono. Human platelets were stimulated for different times (1–12 min) either with 10 μM H_2O_2 (●) or 1 mM H_2O_2 alone (○) (A), or with 1 μM TG + 50 nM Iono in the absence (■) or presence of 1 mM H_2O_2 (□) (B). Actin filament content was determined as described in Section 2. Insets: modification of actin filament content during the initial 60-s period of stimulation with different agents. Results shown are expressed as percentage of control (non-stimulated cells) and are expressed as means ± SEM of 10 independent experiments.

Table 2
Effect of H_2O_2 on the phosphotyrosine content of store depleted human platelets

Stimulatory agent	−BAPTA		+BAPTA	
	Control	H_2O_2	Control	H_2O_2
Basal	1.00 ± 0.00		1.00 ± 0.00	
TG + Iono	1.27 ± 0.11	1.59 ± 0.11*	1.29 ± 0.06	1.51 ± 0.09*
Calcium	1.22 ± 0.08	1.53 ± 0.11*	1.24 ± 0.12	1.49 ± 0.12*

Control and dimethyl BAPTA-loaded platelets were treated in a Ca^{2+}-free medium (200 μM EGTA added) with 1 μM TG + 50 nM Iono in the absence and presence of 1 mM H_2O_2 and 12 min later $CaCl_2$ (1 mM) was added to the medium. Samples were taken from the platelet suspension at 30 s before (basal), 12 min after the addition of TG + Iono in the absence or presence of H_2O_2 and 3 min after $CaCl_2$ addition (calcium). Platelet proteins were analyzed by SDS/10% PAGE and subsequent Western blotting with the specific anti-phosphotyrosine antibody 4G10 as described in Section 2. The results are the integrated attenuances for entire lanes under each condition. Results are expressed as fold increases (means ± SEM for four to six separate experiments) over the integrated attenuance of platelet proteins before Ca^{2+} store depletion.

* $P < 0.05$ compared with the phosphotyrosine content in the absence of H_2O_2.

were incubated for 30 min in the absence or presence of 1 or 10 μg/mL M-2,5-DHC and then treated with 1 mM H_2O_2. We found that treatment with M-2,5-DHC had no effect on resting actin filament content, but resulted in a concentration-dependent inhibition of H_2O_2-induced actin polymerization (Table 3), suggesting that the tyrosine kinase activity is important for actin polymerization induced by high concentrations of H_2O_2.

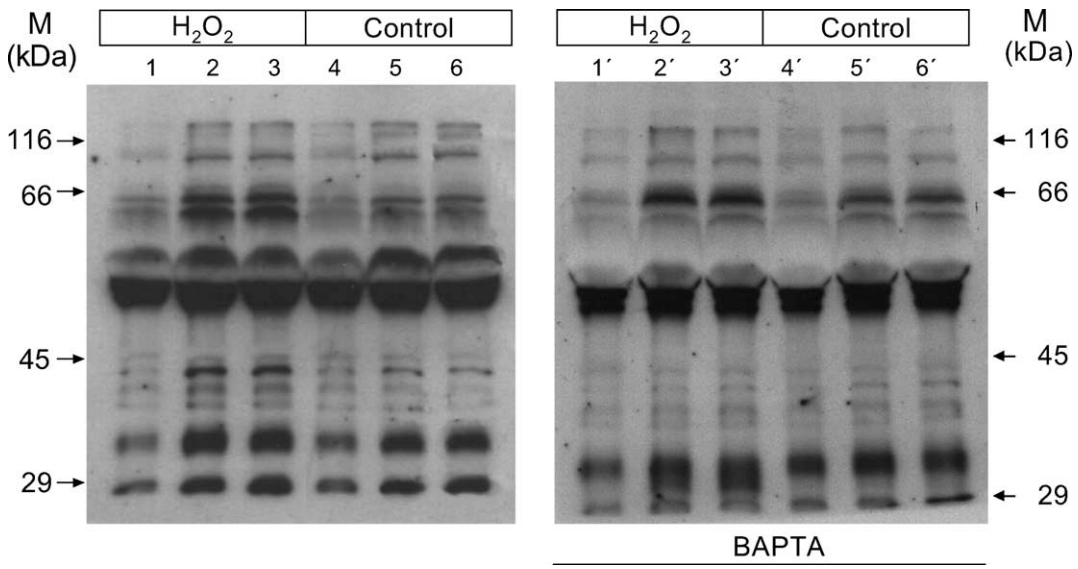

Fig. 6. H_2O_2 increases store depletion-induced tyrosine phosphorylation in control and BAPTA-loaded platelets. Control (left panel) and dimethyl BAPTA-loaded platelets (right panel) were treated in a Ca^{2+}-free medium (200 μM EGTA was added) with 1 μM TG + 50 nM Iono to deplete the intracellular Ca^{2+} stores in the absence and presence of 1 mM H_2O_2, as indicated, and 12 min later $CaCl_2$ (final concentration 1 mM) was added to the medium. Samples were taken from the platelet suspension at 30 s before (lanes 1, 4, 1′ and 4′), 12 min after the addition of TG + Iono in the absence or presence of H_2O_2 (lanes 2, 5, 2′ and 5′) and 3 min after $CaCl_2$ addition (lanes 3, 6, 3′ and 6′). Platelet proteins were analyzed by SDS/10% PAGE and subsequent Western blotting with a specific anti-phosphotyrosine antibody as described in Section 2. Molecular masses ("*M*") 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.

Table 3
Effect of M-2,5-DHC and FTA on H_2O_2-induced increase in the F-actin content in human platelets

Stimulatory agent	Filamentous actin (% of control)			
	Control	M-2,5-DHC		FTA
		1 μg/mL	10 μg/mL	
None	100.0 ± 0.0	99.7 ± 3.3	102.2 ± 6.4	104.6 ± 2.9
H_2O_2	115.6 ± 2.4[§]	105.9 ± 6.2[*]	102.5 ± 7.1[*]	104.5 ± 6.3[*]

Human platelets were suspended in a Ca^{2+}-free medium as described in Section 2. Cells were preincubated for 20 min with 1 or 10 μg/mL M-2,5-DHC, 40 μM FTA or the vehicle (control) and then stimulated with 1 mM H_2O_2. Samples were removed 5 s before and 12 min after the addition of H_2O_2 and F-actin content was determined as described in Section 2. Values given are the F-actin content expressed as a percentage of control and are presented as mean ± SE of 8 to 17 separate determinations.

[*] $P < 0.05$ compared with the actin filament content in the absence of inhibitors.

[§] $P < 0.05$ compared with the actin filament content in non-treated cells.

We further investigated the involvement of proteins of the Ras superfamily of small GTPases, such as Rho and Rac in actin polymerization stimulated by 1 mM H_2O_2. We have recently demonstrated that farnesyl-L-cysteine analogs, such as FTA, which prevent methylation and thus activation of farnesylated Ras proteins, significantly inhibited actin polymerization and SMCE in human platelets [11], which strongly suggest the involvement of Ras superfamily proteins in these processes. Human platelets were incubated for 20 min with FTA (40 μM) and then treated with 1 mM H_2O_2. As shown in Table 3, FTA did not modify the actin filament content of resting platelets but significantly reduced actin polymerization induced by H_2O_2, indicating that Ras superfamily proteins are also important for actin polymerization induced by 1 mM H_2O_2 in human platelets.

3.6. 1 mM H_2O_2 increases the activation of $p60^{src}$ induced by TG + Iono in human platelets

We have recently reported that store depletion induces cytoskeletal association and activation of $p60^{src}$ in non-excitable cells [12,26], which are required for the activation of SMCE [12,26,33]. In the present study, we have investigated the effect of 1 mM H_2O_2 on TG + Iono-induced activation of $p60^{src}$. In a Ca^{2+}-free medium human platelets were treated for 12 min with 1 μM TG + 50 nM Iono and then $CaCl_2$ (1 mM) was added. Samples were taken from the platelet suspension at 30 s before and 12 min after the addition of TG + Iono and 3 min after $CaCl_2$ addition and $p60^{src}$ activity was assessed by Western blotting using a specific anti-phospho-*c*-Src (Y-416) antibody, which specifically detects $p60^{src}$ phosphorylated at Tyr-416, a process indicative of $p60^{src}$ activation [35]. We have found that treatment of platelets with TG + Iono resulted in a significant increase in $p60^{src}$ activity. Consistent with the increase in the phosphotyrosine level, $p60^{src}$ activity was significantly increased when 1 mM H_2O_2 and TG + Iono were added simultaneously. Similar effects were observed when the experiments were performed in platelets heavily loaded with the Ca^{2+} chelator dimethyl BAPTA, suggesting that the increases in

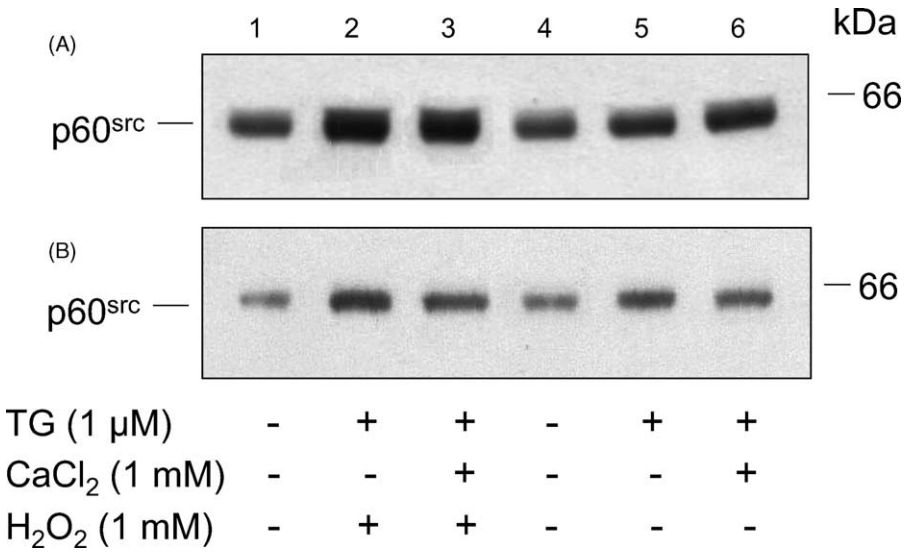

Fig. 7. H_2O_2 increases store depletion-induced $p60^{src}$ activation in control and BAPTA-loaded platelets. Control (upper panel) and dimethyl BAPTA-loaded platelets (lower panel) were treated in a Ca^{2+}-free medium (200 μM EGTA was added) with 1 μM TG + 50 nM Iono to deplete the intracellular Ca^{2+} stores in the absence and presence of 1 mM H_2O_2, as indicated, and 12 min later $CaCl_2$ (final concentration 1 mM) was added to the medium. Samples were taken from the platelet suspension at 30 s before (lanes 1 and 4), 12 min after the addition of TG + Iono in the absence or presence of H_2O_2 (lanes 2 and 5) and 3 min after $CaCl_2$ addition (lanes 3 and 6). Samples were subjected to SDS/10% PAGE and Western blotting with the specific anti-phospho-*c*-Src (Y-416) antibody as described in Section 2. Molecular masses 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.

Table 4
Effect of H_2O_2 on store depletion-induced $p60^{src}$ activation in human platelets

Stimulatory agent	−BAPTA		+BAPTA	
	Control	H_2O_2	Control	H_2O_2
Basal	1.00 ± 0.00		1.00 ± 0.00	
TG + Iono	1.20 ± 0.04	1.29 ± 0.04*	1.24 ± 0.07	1.45 ± 0.12*
Calcium	1.19 ± 0.08	1.50 ± 0.15*	1.19 ± 0.11	1.44 ± 0.05*

Control and dimethyl BAPTA-loaded platelets were treated in a Ca^{2+}-free medium (200 μM EGTA added) with 1 μM TG + 50 nM Iono in the absence and presence of 1 mM H_2O_2 and 12 min later $CaCl_2$ (1 mM) was added to the medium. Samples were taken from the platelet suspension at 30 s before (basal), 12 min after the addition of TG + Iono in the absence or presence of H_2O_2 and 3 min after $CaCl_2$ addition (calcium). Platelet proteins were analyzed by SDS/10% PAGE and subsequent Western blotting with the specific anti-phospho-*c*-Src (Y-416) antibody as described in Section 2. Results are expressed as fold increases (means ± SEM for four to six separate experiments) over "basal" (non-treated cells) values.

* $P < 0.05$ compared with the $p60^{src}$ activity in the absence of H_2O_2.

$p60^{src}$ activity were not due to the elevation in $[Ca^{2+}]_i$ induced by store depletion (Fig. 7 and Table 4, $P < 0.05$, N = 4–6).

4. Discussion

The actin cytoskeleton is a complex and dynamic structure organized into two major structures: a membrane-associated cytoskeleton and a cytosolic network. Many signal transduction molecules induce reorganization of the actin cytoskeleton, which is involved in a wide range of cellular processes, including cell shape and motility [36], intracellular trafficking [37], G-protein signaling [38] and secretion [31]. Recently, a role for the actin cytoskeleton in SMCE has received support from studies which show that the activation of SMCE shares properties with secretion [4–7,26,39]. This secretion-like coupling model suggests that Ca^{2+} store depletion leads the trafficking of portions of the ER towards the PM to facilitate the coupling between proteins in both membranes, where the actin cytoskeleton plays an important regulatory role. In support of this hypothesis, recent studies have reported interaction between calcium channels in the PM and IP_3 receptors in the ER [7,40–42].

Our observations indicate that platelet exposure to low H_2O_2 concentrations favor Ca^{2+} release from intracellular stores and subsequently, SMCE. In agreement with the mechanism involved in agonist-induced SMCE [43], we found a positive correlation between Ca^{2+} release and entry at 10 or 100 μM H_2O_2. Consistent with this, H_2O_2 (10 μM) induced a temporal reorganization of the actin cytoskeleton that was similar to that induced by store depletion by TG + Iono or by secretagogues in pancreatic acinar cells [15,31,44]. This actin remodeling consists of an initial net depolymerization followed by a net increase in the actin filament content. Our observations are consistent with a secretion-based coupling model to explain the activation of SMCE, where, as for secretion, an initial actin depolymerization should occur at the cell periphery, where cortical actin filaments prevent constitutive SMCE activation blocking the approach of portions of the ER to the PM [4–7]. The correlation between Ca^{2+} release and entry and the similar actin reorganization induced by H_2O_2 and TG + Iono, suggests that low concentrations of H_2O_2 might act as physiological molecules in human platelets, as previously reported [20,21].

We have found that H_2O_2 exerts a biphasic concentration-dependent stimulation of SMCE, so that the ability of H_2O_2 to induce SMCE decreases at higher concentrations. In addition, and in agreement with previous studies [22], 1 mM H_2O_2 reduced both the activation and maintenance of SMCE stimulated by agonists. These findings are consistent with the lack of an initial net depolymerization in the temporal sequence of actin reorganization induced by 1 mM H_2O_2. In addition, H_2O_2 (1 mM) clearly modifies the reorganization pattern induced by TG + Iono, which might explain the decrease in SMCE induced by this concentration of H_2O_2, as it does in secretion, where absence of initial depolymerization inhibits exocytosis in pancreatic acini [15]. To further explore whether the inhibitory effect of H_2O_2 is mediated by actin polymerization we tested the effect of Cyt D. We have previously reported that 10 μM Cyt D exerted a biphasic and time-dependent effect on SMCE in platelets. One-minute treatment with Cyt D induced a small increase in SMCE while 20- or 40-min treatment with Cyt D reduced it and no change was observed after 10 min of preincubation with this agent [11]. Hence, we used the treatment for 10 min with Cyt D to inhibit actin polymerization but inducing minimal effects on Ca^{2+} entry. Treatment of platelets with Cyt D for 10 min abolished H_2O_2-induced actin polymerization either when added alone or in combination with TG + Iono or thrombin. Interestingly, blockade of H_2O_2-evoked actin polymerization prevented the inhibitory effect of H_2O_2 on SMCE, which strongly suggests that, at high concentrations, H_2O_2 stimulates an exacerbated actin polymerization which disrupts the dynamic equilibrium between polymerizing and depolymerizing forces in favor of the latter and leads to inhibition of SMCE in these cells.

In FRTL-5 cells ROS-induced inhibition of Ca^{2+} entry has been attributed to an increase in the activity of the plasma membrane Ca^{2+} ATPase (PMCA) [22,45]. In contrast, we have previously observed that 1 mM H_2O_2 reduced Ca^{2+} extrusion by the PMCA in human platelets [46], which is the main mechanism for Ca^{2+} efflux at the $[Ca^{2+}]_i$ working range [47].

Given that the actin cytoskeleton mediates the inhibitory effects of H_2O_2 on SMCE, the nature of the mechanism leading to actin polymerization remains to be elucidated, although there are two possibilities, either a direct oxidation of actin itself and actin-binding proteins or an effect

mediated by a cytoskeleton-related second messenger. Different studies have demonstrated oxidation of the sulfydryl group at Cys^{374} located in the C-terminal segment of actin [14]. This segment is very important for the stability of actin filaments and the association of several actin-binding proteins, which is essential to modulate the actin polymerization dynamics [14,48]. In addition, several molecules have been shown to participate in actin remodeling, including proteins of the Ras superfamily and tyrosine kinases [9–12]. In the present study, we provide evidences in favor of the involvement of Ras superfamily proteins in H_2O_2-induced actin polymerization by using FTA, a farnesyl cysteine analog that prevents post-translational processing and membrane association of small GTP-binding proteins of the Ras superfamily, which results in inhibition of their activation [11,50,51].

It is well known that H_2O_2 activates tyrosine kinases in different cell types [49]. Here we show that H_2O_2 (1 mM) potentiates store depletion-induced increase in the phosphotyrosine level even in the absence of any $[Ca^{2+}]_i$ elevation indicating the lack of dependence of rises in $[Ca^{2+}]_i$. One of the kinases activated by treatment with high concentrations of H_2O_2 is the Src family protein $p60^{src}$, activated by store depletion, as previously described by us [26], and whose activity was further increased by exposure to 1 mM H_2O_2. Recent studies have shown that $p60^{src}$ is required for the activation but not the maintenance of SMCE in different cell types [12,26,33]. Although the nature of this mechanism is poorly understood, it does not appear to be mediated by actin polymerization, which instead seems to be required for the cytoskeletal association and activation of $p60^{src}$ [12,26]. This fact, together with the lack of involvement in the maintenance of SMCE, also inhibited by 1 mM H_2O_2 (see Fig. 2F and G) suggests that $p60^{src}$ activation by H_2O_2 is not involved in the inhibitory effect of this oxidant. To investigate whether tyrosine phosphorylation is required for H_2O_2-induced actin polymerization, we studied the effect of M-2,5-DHC. Our results indicate that in platelets treated with M-2,5-DHC, the ability of H_2O_2 to induce actin polymerization was significantly reduced, suggesting a role for tyrosine kinases, different from $p60^{src}$, in actin polymerization evoked by high concentrations of H_2O_2 in these cells. This effect of H_2O_2 on tyrosine phosphorylation might be strengthen by the recently described ability of H_2O_2 to inactivate certain tyrosine phosphatases [52].

In conclusion we suggest that H_2O_2 plays a dual role in SMCE in human platelets, both as a messenger molecule that induces store depletion which might be linked to SMCE by the described physiological mechanisms (for review see [53]), and as injurious by-products of cellular metabolism, which alter the dynamics of polymerization–depolymerization, by a mechanism involving the activation of tyrosine kinases and Ras superfamily proteins, that lead to inhibition of SMCE in these cells.

Acknowledgments

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