Differential involvement of thrombin receptors in Ca^{2+} release from two different intracellular stores in human platelets

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Physiological agonists increase cytosolic free Ca^{2+} concentration to regulate a number of cellular processes. The platelet thrombin receptors, PAR (protease-activated receptor) 1 PAR-4 and GPIb-IX-V (glycoprotein Ib-IX-V) have been described as potential contributors of thrombin-induced platelet aggregation. Platelets present two separate Ca^{2+} stores, the DTS (dense tubular system) and acidic organelles, differentiated by the distinct sensitivity of their respective SERCAs (sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPases) to TG (thapsigargin) and TBHQ [2,5-di-(tert-butyl)-1,4-hydroquinone]. However, the involvement of the thrombin receptors in Ca^{2+} release from each Ca^{2+} store remains unknown. Depletion of the DTS using ADP, which releases Ca^{2+} solely from the DTS, in combination with 10 nM TG, to selectively inhibit SERCA2 located on the DTS reduced Ca^{2+} release evoked by the PAR-1 agonist, SFLLRN, and the PAR-4 agonist, AYPGKF, by 80 and 50 % respectively. Desensitization of PAR-1 and PAR-4 or pre-treatment with the PAR-1 and PAR-4 antagonists SCH 79797 and tcY-NH_2 reduced Ca^{2+} mobilization induced by thrombin, and depletion of the DTS after desensitization or blockade of PAR-1 and PAR-4 had no significant effect on Ca^{2+} release stimulated by thrombin through the GPIb-IX-V receptor. Converse experiments showed that depletion of the acidic stores using TBHQ reduced Ca^{2+} release evoked by SFLLRN or AYPGKF, by 20 and 50 % respectively, and abolished thrombin-stimulated Ca^{2+} release through the GPIb-IX-V receptor when PAR-1 and PAR-4 had been desensitized or blocked. Our results indicate that thrombin-induced activation of PAR-1 and PAR-4 evokes Ca^{2+} release from both Ca^{2+} stores, while activation of GPIb-IX-V by thrombin releases Ca^{2+} solely from the acidic compartments in human platelets.

Key words: acidic organelles, calcium, dense tubular system (DST), platelet, protease-activated receptor 1 (PAR-1), thrombin.

INTRODUCTION

Human platelets possess two separate agonist-releasable Ca^{2+} stores differentiated by the distinct sensitivity of the SERCA (sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase) isoforms located on each store to TG (thapsigargin) and TBHQ [2,5-di-(tert-butyl)-1,4-hydroquinone]. The major store has long been identified as the DTS (dense tubular system). This store expresses SERCA2b, which is inhibited by low concentrations of TG and is insensitive to TBHQ [1,2]. The acidic nature of the second store has recently been identified [3] and expresses SERCA3, which shows a lower sensitivity to TG but is sensitive to TBHQ [1,3–5]. Both stores are sensitive to the physiological agonist thrombin [6].

Thrombin stimulates human platelets by activation of two G-protein-coupled protease-activated receptors, PAR-1 and PAR-4 [7–9] and the leucine-rich glycoprotein receptor GPIb-IX-V [10,11]. Both PAR (protease-activated receptor) 1 and PAR-4 are cleaved by thrombin at specific sites in the extracellular domain. Removal of this peptide results in a new N-terminal sequence that acts as a 'tethered ligand' and initiates transmembrane signalling [9,10]. Peptides reproducing the sequence of the new N-terminal sequence of activated PAR-1 and PAR-4 receptors, such as SFLLRN and AYPGKF, are potent and selective activators of PAR-1 and PAR-4 respectively, and trigger thrombin-evoked platelet responses [8,11–13].

GPIb-IX-V (glycoprotein Ib-IX-V) binds thrombin with high affinity and may contribute to the activation of platelets [14,15]. Platelet activation by low concentrations of thrombin is impaired by antibodies against GPIb-IX-V [16]. The functional significance of the binding of thrombin to platelet GPIb-IX-V is not fully established but it has been reported that binding of thrombin to GPIb-IX-V may initiate a new mechanism for platelet aggregation independent of PARs [15,16]. Platelet stimulation with thrombin upon PAR-1 and PAR-4 desensitization activates a number of intracellular pathways, including phosphorylation of mitogen-activated protein kinase and activation of the Rho-dependent kinase p160ROCK [9,16], which suggests a role for GPIb-IX-V in thrombin-induced intracellular signalling and platelet activation [17].

In the present study, we have investigated the relative contribution of the two Ca^{2+} compartments present in human platelets in Ca^{2+} release induced by activation of the thrombin receptors PAR-1, PAR-4 and GPIb-IX-V. We have found that selective activation of either PAR-1 or PAR-4 releases Ca^{2+} from both stores, although the contribution of each store in Ca^{2+} mobilization induced by PAR-1 or PAR-4 is clearly different. In contrast, Ca^{2+} release upon activation of GPIb-IX-V is entirely dependent on the acidic compartments in human platelets.

MATERIAL AND METHODS

Materials

Fura-2/AM (Fura-2 acetoxymethyl ester), and calcein were from Molecular Probes (Leiden, The Netherlands). Apyrase (grade V),

Abbreviations used: $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; Cyt D, cytochalasin D; DTS, dense tubular system; Fura-2/AM, Fura-2 acetoxymethyl ester; GPIb-IX-V, glycoprotein Ib-IX-V; HBS, Hepes-buffered saline; Iono, ionomycin; NAADP, nicotinic acid-adenine dinucleotide phosphate; PAR, protease-activated receptor; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; SOCE, store-operated calcium entry; TBHQ, 2,5-di-(tert-butyl)-1,4-hydroquinone; TG, thapsigargin.

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aspirin, thrombin, ADP, BSA, Iono (ionomycin) and TG were from Sigma (Madrid, Spain). SFLLRN and AYPGKF were from Bachem (St. Helens Merseyside, U.K.). TcY-NH_2 and SCH 79797 were from Tocris (Bristol, U.K.). TBHQ was from Alexis (Nottingham, U.K.). All other reagents were of analytical grade.

Platelet preparation

Fura-2-loaded human platelets were prepared as described previously [18], as approved by Local Ethical Committees. Briefly, blood was obtained from healthy volunteers and mixed with one-sixth volume of acid/citrate dextrose anti-coagulant 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 then 100 μM aspirin and 40 μg/ml apyrase were added. Platelet-rich plasma was incubated at 37 °C 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 (Hepes-buffered saline) containing 145 mM NaCl, 10 mM Hepes (pH 7.45), 10 mM D-glucose, 5 mM KCl and 1 mM $MgSO_4$, and supplemented with 0.1 % BSA and 40 μg/ml apyrase.

Cell viability

Calcein and Trypan Blue were used to assess cell viability. For calcein loading, resting cells, or treated with inhibitors for the times indicated, were incubated for 30 min with 5 μM calcein-acetoxymethyl ester at 37 °C, centrifuged (at 350 ***g***) and the pellet was resuspended in fresh HBS. Fluorescence was recorded from 2 ml aliquots using a Shimadzu spectrophotometer (Shimadzu, Japan). Samples were excited at 494 nm and the resulting fluorescence was measured at 535 nm. The calcein fluorescence remaining in the cells after treatment with the inhibitors used was the same as in control, suggesting that under our conditions there was no cellular damage. The results obtained with calcein were confirmed using the Trypan Blue exclusion technique. Of the cells, 95 % were viable after treatment with the inhibitors, similar to that observed in our resting platelet suspensions.

Measurement of $[Ca^{2+}]_c$ (cytosolic free calcium concentration)

Fluorescence was recorded from 2 ml aliquots of magnetically stirred cell suspensions (10^8 cells/ml) at 37 °C using a Fluorescence spectrophotometer (Varian Ltd., Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were monitored using the Fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. [19]. Thrombin, TG and Iono, SFLLRN or AYPGKF-induced Ca^{2+} release was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after their addition by the removal of a sample every 1 s and was expressed as nM · s [20].

Statistical analysis

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

RESULTS

Thrombin releases Ca^{2+} from the DTS and acidic organelles in human platelets

Fura-2-loaded, aspirin-treated human platelets were used to assess Ca^{2+} release from the intracellular stores evoked by different concentrations (0.01–0.5 units/ml) of the physiological agonist thrombin. In a Ca^{2+}-free medium (100 μM EGTA was added) platelet stimulation with thrombin induced a transient increase

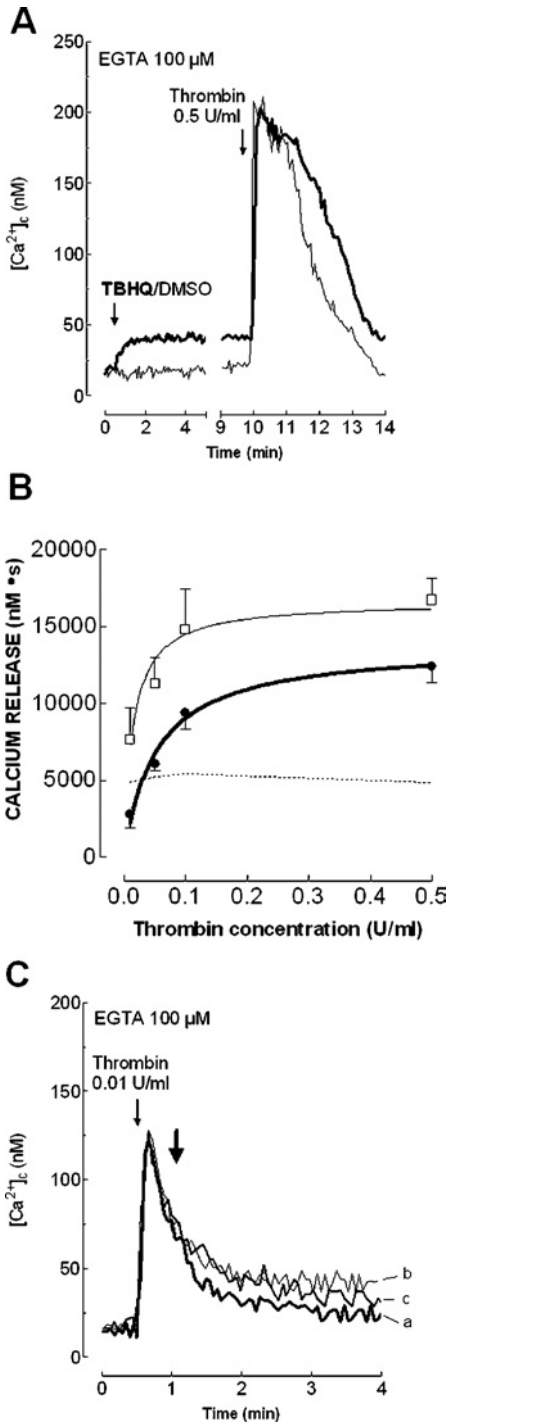

Figure 1 Thrombin-induced Ca^{2+} release from TBHQ-sensitive and -insensitive intracellular stores

(**A**) Fura 2-loaded human platelets were treated in Ca^{2+}-free medium (100 μM EGTA was added to remove Ca^{2+}) for 9 min at 37 °C with 20 μM TBHQ (bold line) or with DMSO (vehicle; thin line). Cells were then stimulated with 0.5 units/ml of thrombin. Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of six independent experiments. (**B**) Results represent Ca^{2+} release induced by increasing concentrations of thrombin in the absence (□) or presence (●) of TBHQ, and are presented as means ± S.E.M. The dashed line represents the difference between both curves. (**C**) Fura 2-loaded human platelets were stimulated in Ca^{2+}-free medium (100 μM EGTA was added to remove Ca^{2+}) at 37 °C with 0.01 units/ml of thrombin alone (a) or in combination with 20 μM TBHQ (b) or were stimulated with 0.01 units/ml of thrombin and 30 s later TBHQ was added as indicated by the thick arrow (c). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of four independent experiments.

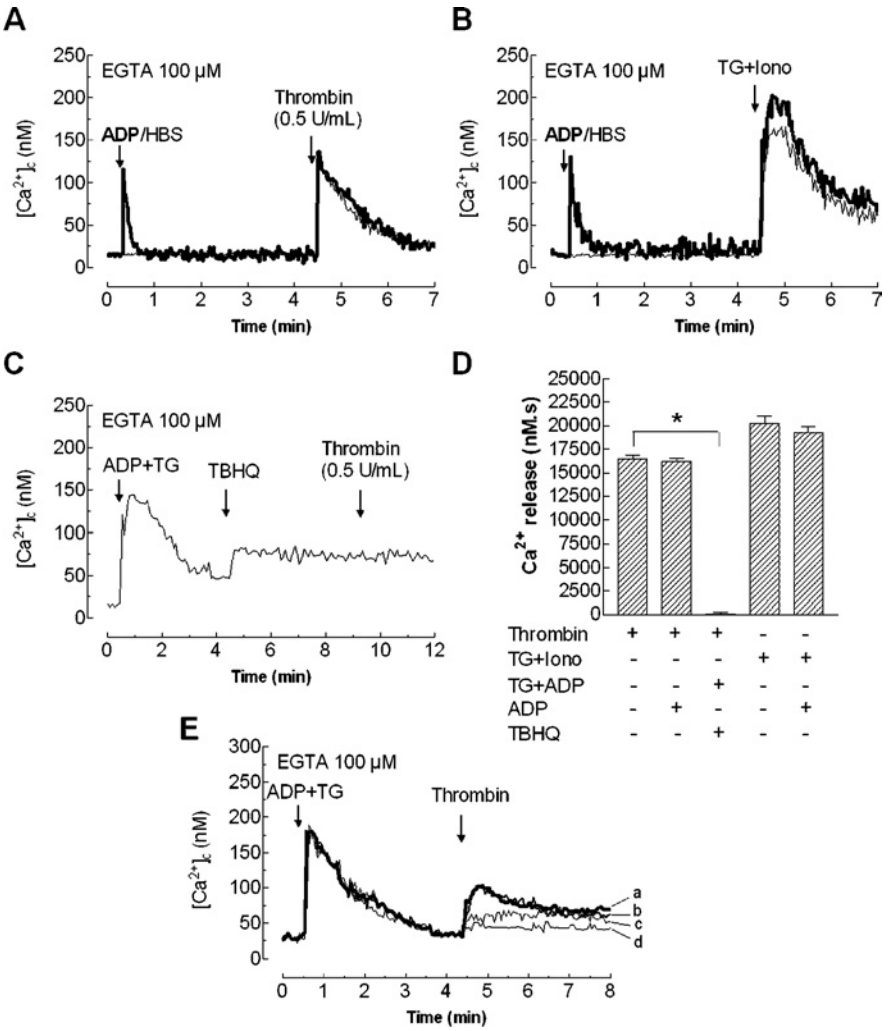

Figure 2 Depletion of the DTS and acidic stores impair thrombin-induced Ca^{2+} release in human platelets

(**A** and **B**) Fura 2-loaded human platelets, resuspended in Ca^{2+}-free medium (100 μM EGTA was added at the time of experiment), were treated with 10 μM ADP (bold trace) or HBS (vehicle; thin trace) and 4 min later 0.5 units/ml of thrombin (**A**) or 1 μM TG and 50 nM Iono (**B**) were added to release Ca^{2+} from the stores. (**C**) Fura 2-loaded human platelets were treated in Ca^{2+}-free medium with 10 μM ADP in combination with 10 nM TG to deplete the DTS, and 4 min later 20 μM TBHQ was added to discharge the acidic compartments. After a further 5 min, cells were stimulated with 0.5 units/ml of thrombin. Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of six independent experiments. (**D**) Histograms represent Ca^{2+} release stimulated by thrombin or TG and Iono under different experimental conditions as indicated. Results are presented as means $\pm$ S.E.M. $^*P < 0.01$. (**E**) Fura 2-loaded human platelets were treated in Ca^{2+}-free medium with 10 μM ADP in combination with 10 nM TG to deplete the DTS, and 4 min later the cells were stimulated with thrombin (0.5, 0.01, 0.005 or 0.001 units/ml; traces a, b, c and d respectively). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of four independent experiments.

in $[Ca^{2+}]_c$ (Figure 1A), which, as expected, was found to be concentration dependent (Figure 1B). Treatment of platelets with 20 μM TBHQ (to allow depletion of the acidic store [3,6]) 9 min prior to stimulation with thrombin significantly decreased Ca^{2+} mobilization by thrombin at concentrations between 0.01 and 0.5 units/ml to a similar extent (Figure 1B), which suggests that concentrations as low as 0.01 units/ml of thrombin discharge the acidic stores in platelets. To confirm this issue we have performed a series of experiments that showed that treatment of platelets with 0.01 units/ml of thrombin alone or in combination with 20 μM TBHQ and this resulted in a similar increase in $[Ca^{2+}]_c$ (Figure 1C; $n = 4$). The initial peak elevation in $[Ca^{2+}]_c$ was not significantly different in cells treated with thrombin alone and in combination with TBHQ (126 ± 8 and 125 ± 10 nM respectively; $P > 0.05$). It should be noted that the return of $[Ca^{2+}]_c$ to basal levels after cell stimulation was slower in the presence of TBHQ, which is likely to be due to the inhibition of SERCA3, and therefore impairment of the reuptake of Ca^{2+} into the TBHQ-sensitive store. In addition, we have investigated whether pre-treatment with 0.01 units/ml of thrombin prevented the subsequent response to TBHQ. To minimize the possible effect of refilling of the TBHQ-sensitive store after stimulation with thrombin, TBHQ was added just 30 s after the addition of the agonist. Our results indicate that treatment of platelets with 0.01 units/ml of thrombin abolished TBHQ-induced Ca^{2+} release from the TBHQ-sensitive store (Figure 1C). Both in the absence and presence of TBHQ, thrombin-evoked Ca^{2+} release was concentration-dependent and followed a similar pattern (Figure 1B), suggesting that thrombin induces a concentration-dependent Ca^{2+} release from the DTS in human platelets.

Thrombin receptors PAR-1 and PAR-4 release Ca^{2+} differentially from the DTS in human platelets

To achieve depletion of the DTS, cells were treated with a low concentration of TG (10 nM), to inhibit specifically SERCA2b,

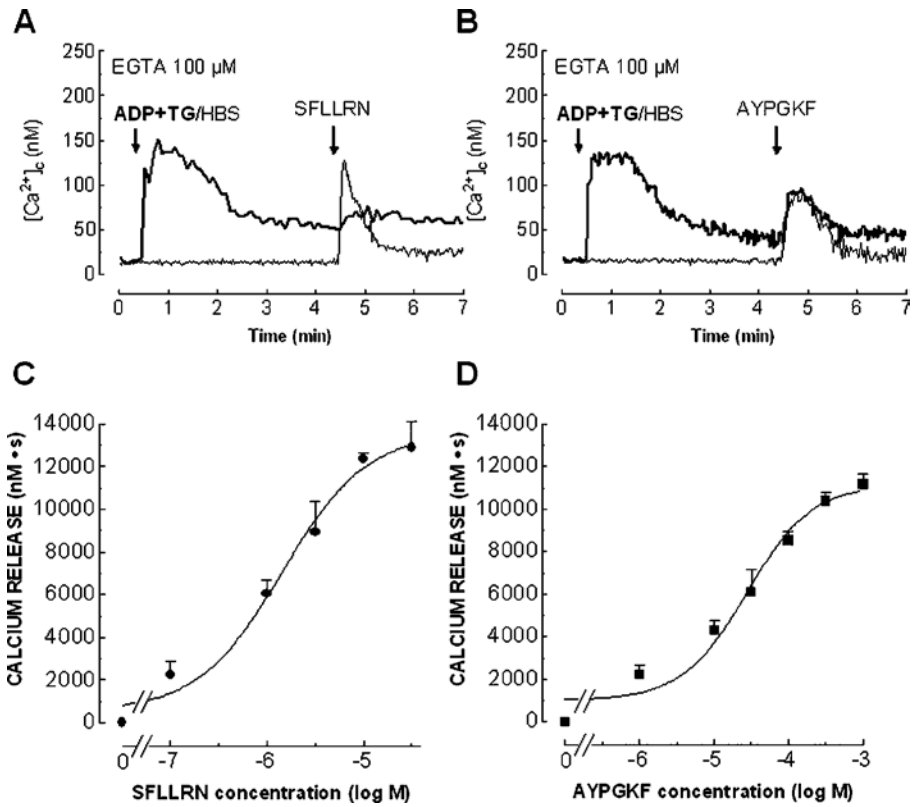

Figure 3 Effect of DTS depletion on SFLLRN- or AYPGKF-induced Ca^{2+} release in human platelets

(**A** and **B**) Fura 2-loaded human platelets, resuspended in Ca^{2+}-free medium (100 μM EGTA was added at the time of experiment), were treated with 10 μM ADP in combination with 10 nM TG (bold trace) or the HBS (vehicle; thin trace) and4 min later 10 μM SFLLRN (**A**) or 500 μM AYPGKF (**B**) were added to release Ca^{2+} from the stores. Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of six to eight independent experiments. (**C** and **D**) Fura 2-loaded human platelets, resuspended in Ca^{2+}-free medium were stimulated with various concentration of SFLLRN (**C**) or AYPGKF (**D**). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Ca^{2+} release was estimated as described in Material and methods. Results are presented as means $\pm$ S.E.M. of four independent experiments.

which is present on the membrane of the DTS and shows high sensitivity to TG [2,21,22], in combination with 10 μM ADP, which selectively releases Ca^{2+} from the DTS without affecting the acidic stores [6] and is required to induce rapid discharge of the store. In a Ca^{2+}-free medium, platelet stimulation with ADP did not alter subsequent Ca^{2+}-release induced by 0.5 units/ml of thrombin, suggesting that the use of ADP did not desensitize the thrombin receptors (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of thrombin was 16433 ± 478 and 16215 ± 341 nM · s in the absence or presence of ADP; Figures 2A and 2D; $P > 0.05$; $n = 6$). In addition, treatment with ADP did not significantly reduce Ca^{2+}-release induced by addition of 1 μM TG in combination with 50 nM Iono to fully deplete the intracellular Ca^{2+} stores (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of TG and Iono was 20226 ± 784 and 19261 ± 657 nM · s in the absence and presence of ADP respectively), which indicates that Ca^{2+} release induced by ADP is rapidly taken-up again into the DTS and supports the lack of effect of ADP on thrombin-evoked response (Figures 2B and 2D; $P > 0.05$; $n = 6$).

In order to investigate whether the treatment with ADP + TG is effectively inducing depletion of the DTS, we tested the effect of 0.5 units/ml thrombin in platelets where the DTS had been discharged by treatment with ADP + TG, followed by depletion of the acidic stores by addition of 20 μM TBHQ. As depicted in Figures 2(C) and 2(D), thrombin-evoked Ca^{2+} release was abolished by prior treatment with ADP + TG and TBHQ, which indicates that depletion of the DTS by ADP + TG was effective ($P < 0.01$; $n = 6$).

We have reported above that thrombin-evoked Ca^{2+} release from the DTS is concentration dependent. Treatment of platelets with thrombin, which had been pre-treated with 10 μM ADP and 10 nM TG to deplete the DTS, at concentrations of 0.001 to 0.5 units/ml, induced a concentration dependent Ca^{2+} release from the TBHQ-sensitive store, suggesting that Ca^{2+} release from this store by thrombin was also concentration dependent (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of 0.001, 0.005, 0.01 and 0.5 units/ml of thrombin was 920 ± 200, 3693 ± 996, 6427 ± 764 and 6500 ± 850 nM · s respectively; Figure 2E; $n = 4$).

As shown in Figure 3(A), in a Ca^{2+}-free medium, treatment of platelets with the PAR-1 agonist, SFLLRN (10 μM), evoked a rapid and transient increase in $[Ca^{2+}]_c$. Selective stimulation of PAR-4 with the peptide AYPGKF (500 μM) also induced a transient rise in $[Ca^{2+}]_c$ that was found to be smaller in magnitude but more sustained than the rise in $[Ca^{2+}]_c$ observed after treatment with SFLLRN (Figure 3B).

Treatment of human platelets with increasing concentrations of SFLLRN (0.1–30 μM) or AYPGKF (1–1000 μM) induced Ca^{2+} release from intracellular stores in a concentration-dependent manner, reaching a maximum at 30 μM for SFLLRN and 1000 μM for AYPGKF and an EC_{50} of 1.4 and 27 μM respectively (Figures 3C and 3D; $n = 4$). During the performance of the present study we have used SFLLRN and AYPGKF at the concentrations

10 and 500 μM, respectively, which showed a similar effect on Ca^{2+} release compared with the maximal concentrations of the agonists (Figures 3C and 3D), and have been used by others previously [23,24].

Ca^{2+} release evoked by 10 μM SFLLRN was significantly reduced by 80 ± 10 % when the DTS had been previously depleted by treatment with 10 μM ADP and 10 nM TG (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of SFLLRN taking a sample every 1 s was 12573 ± 3774 and 2517 ± 341 nM · s in the absence or presence of ADP + TG; Figure 3A; $n = 6$; $P < 0.01$). In contrast, depletion of the DTS by treatment with ADP and TG reduced Ca^{2+} release evoked by 500 μM AYPGKF by only 50 ± 7 % (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of AYPGKF taking a sample every second was 10231 ± 1434 and 5254 ± 765 nM · s in the absence or presence of ADP + TG; Figure 3B; $n = 8$; $P < 0.01$).

Thrombin-evoked Ca^{2+} release through the GPIb-IX-V receptor is independent of the DTS

Characterization of Ca^{2+} mobilization upon activation of the GPIb-IX-V receptor by thrombin was investigated by desensitizing the PAR-1 and PAR-4 receptors. Desensitization was achieved by platelet treatment with 10 μM SFLLRN in combination with 750 μM AYPGKF. As shown in Figure 4(A), desensitization of PAR-1 and PAR-4 was confirmed by the inability of SFLLRN and AYPGKF to increase $[Ca^{2+}]_c$ in PAR-desensitized platelets. Subsequent addition of 1 μM TG in combination with 50 nM Iono to fully discharge the intracellular Ca^{2+} stores confirmed that the lack of effect of SFLLRN and AYPGKF was not due to previous store depletion [the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of TG and Iono was 19098 ± 906 nM · s, similar to that found in control cells, as shown in Figure 2(D); $n = 6$]. Preliminary experiments showed that PAR-4 desensitization was not completely achieved by 500 μM AYPGKF and a number of platelet preparations still responded slightly to a second addition of the peptide; however, no response was observed when 750 μM AYPGKF was used.

Platelet stimulation with 0.5 units/ml of thrombin when PAR-1 and PAR-4 receptors had been desensitized induced a transient increase in $[Ca^{2+}]_c$ that was significantly smaller than that observed in control cells (not pre-treated with SFLLRN and AYPGKF; Figure 4B; $n = 6$). The integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of thrombin was 16730 ± 1430 and 3462 ± 459 nM · s in control and PAR-desensitized platelets respectively. In cells where PAR-1 and PAR-4 had been desensitized, depletion of the DTS using ADP and TG had no significant effect on thrombin-evoked Ca^{2+} release (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of thrombin was 3398 ± 406 nM · s in PAR-desensitized cells treated with ADP + TG; Figure 4C; $P > 0.05$; $n = 6$).

Thrombin-evoked Ca^{2+} release by activation of PAR-1, PAR-4 and GPIb-IX-V receptors is dependent on the acidic stores

As shown previously [3,6] and depicted in Figure 2(C), treatment of platelets with 20 μM TBHQ fully depletes the acidic compartments. In Ca^{2+}-free medium, selective stimulation of PAR-1 with the peptide SFLLRN (10 μM) induced a transient rise in $[Ca^{2+}]_c$ that was reduced by pre-treatment with TBHQ by 20 ± 4 % (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of SFLLRN was 11786 ± 1293 and 9402 ± 2031 nM · s in the absence or presence of TBHQ; Figure 5A; $P < 0.05$; $n = 6$). As observed by depletion of the DTS, discharge of the acidic stores with TBHQ also reduced the elevation in $[Ca^{2+}]_c$ induced by the PAR-4 agonist peptide AYPGKF (500 μM) by 40 ± 4 % (the integral of the rise in $[Ca^{2+}]_c$ for 2.6 min after the addition of AYPGKF was 10277 ± 2798 and 6533 ± 1147 nM · s in the absence or presence of TBHQ; Figure 5B; $n = 6$; $P < 0.05$). These findings suggest that PAR-1- and PAR-4-evoked Ca^{2+} release from the acidic stores is complementary with Ca^{2+} mobilization from the DTS.

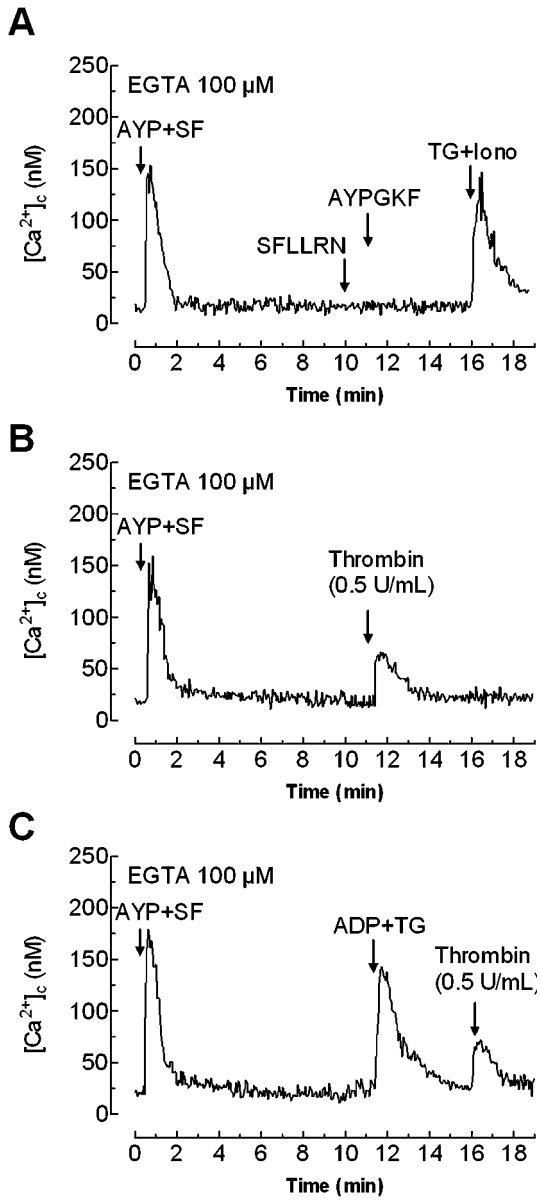

Figure 4 Effect of DTS depletion on thrombin-induced Ca^{2+} release by activation of the GPIb-IX-V receptor in human platelets

(**A**) Fura 2-loaded human platelets were treated in Ca^{2+}-free medium (100 μM EGTA was added at the time of experiment) with 10 μM SFLLRN in combination with 750 μM AYPGKF to desensitize PAR-1 and PAR-4. Cells were treated 9.5 min later with 10 μM SFLLRN followed by the addition of 500 μM AYPGKF 1.5 min later. TG (1 μM) and Iono (50 nM) were added 5 min later to deplete the intracellular Ca^{2+} stores. (**B** and **C**) Fura 2-loaded human platelets were treated in Ca^{2+}-free medium with 10 μM SFLLRN in combination with 750 μM AYPGKF to desensitize PAR-1 and PAR-4. Cells were then either stimulated with 0.5 units/ml of thrombin (**B**) or with 10 μM ADP in combination with 10 nM TG to deplete the DTS, followed by addition of 0.5 units/ml of thrombin 5 min later (**C**). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of six independent experiments.

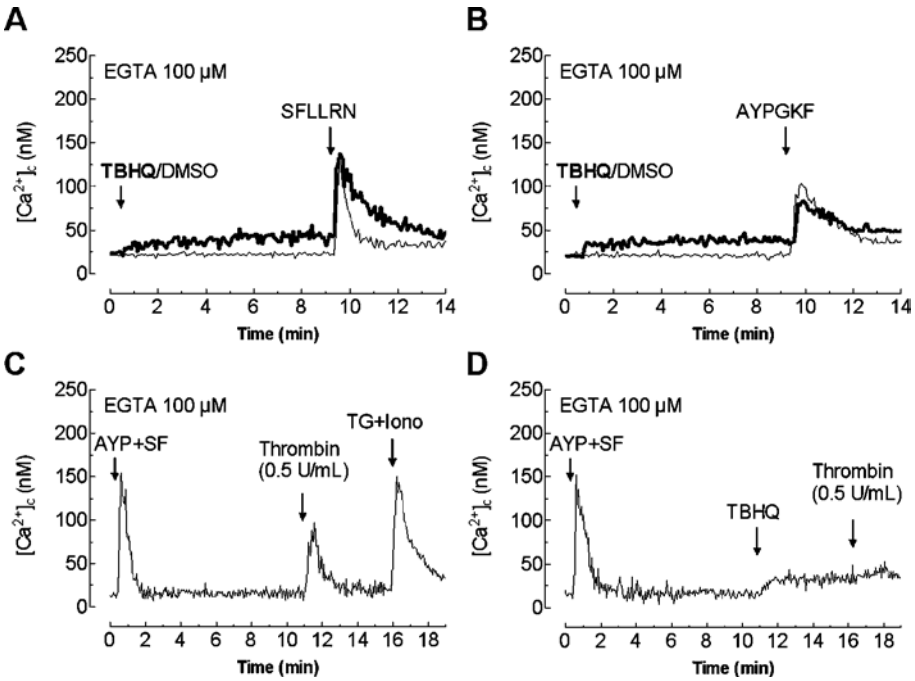

Figure 5 Effect of depletion of the acidic stores on Ca^{2+} release induced by occupation of thrombin receptors in human platelets

(**A** and **B**) Fura 2-loaded human platelets, resuspended in Ca^{2+}-free medium (100 μM EGTA was added at the time of experiment), were treated with 20 μM TBHQ (bold trace) or DMSO (vehicle; thin trace) and 9 min later 10 μM SFLLRN (**A**) or 500 μM AYPGKF (**B**) were added to release Ca^{2+} from the stores. (**C** and **D**) Fura 2-loaded human platelets were treated in a Ca^{2+}-free medium with SFLLRN (10 μM) in combination with AYPGKF (750 μM) to desensitize PAR-1 and PAR-4. After a further 11 min, the cells were either stimulated with 0.5 units/ml of thrombin followed by addition of 1 μM TG and 50 nM Iono (**C**) or were treated with 20 μM TBHQ to discharge the acidic compartments followed by addition of 0.5 units/ml of thrombin (**D**). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of six independent experiments.

Finally, we have investigated whether Ca^{2+} mobilization by thrombin through the GPIb-IX-V receptor depends on the acidic stores. PAR-1 and PAR-4 were desensitized as described above and the effect of thrombin on Ca^{2+} release was tested. As reported above, the rise in $[Ca^{2+}]_c$ under these conditions was much smaller compared with cells where PAR-1 and PAR-4 were active (compare Figure 5C with Figure 1A). The reduction in thrombin-evoked Ca^{2+} mobilization was not due to store depletion, since treatment with 1 μM TG and 50 nM Iono, added to fully deplete the Ca^{2+} stores, was able to increase $[Ca^{2+}]_c$. In cells where PAR-1 and PAR-4 had been desensitized, depletion of the acidic store using TBHQ almost completely inhibited thrombin-evoked Ca^{2+} release (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of thrombin was 3462 ± 459 and 684 ± 35 nM · s in PAR-desensitized cells in the absence or presence of TBHQ; compare Figure 5D with Figure 5C; $n = 6$; $P < 0.01$).

These findings were confirmed using the PAR-1 and PAR-4 antagonists SCH 79797 (3 μM) and tcY-NH_2 (400 μM) respectively, as reported previously [13,25]. We found that pre-treatment of platelets for 30 min with 3 μM SCH 79797 or 400 μM tcY-NH_2 abolished Ca^{2+} release evoked by 10 μM SFLLRN or 500 μM AYPGKF respectively (results not shown). As shown in Figure 6(A), in cells pre-treated with the PAR antagonists, thrombin-evoked Ca^{2+} release was significantly reduced as shown by PAR desensitization (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of thrombin to cells pre-treated with PAR antagonists was 3389 ± 402 nM · s). The remaining thrombin-induced response was not significantly reduced by treatment with ADP and TG (the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after the addition of thrombin was 3402 ± 389 nM · s; Figure 6B; $P > 0.05$; $n = 4$) but was abolished when cells were stimulated in the presence of TBHQ, which further suggest that Ca^{2+} release from the intracellular stores evoked by activation of the GPIb-IX-V receptor depends on the acidic stores (Figure 6C; $P < 0.01$; $n = 4$).

DISCUSSION

Platelet stimulation with the physiological agonist thrombin induces release of Ca^{2+} from the intracellular agonist-releasable stores. Two separate stores have been described in human platelets based on pharmacological, functional and immunological studies [1–6]. The DTS, the analogue of the endoplasmic reticulum in platelets, is a major Ca^{2+} store [6,26] releasable by platelet agonists such as thrombin, ADP or vasopressin through Ins(1,4,5)P_3 generation [6]. The second store has been identified as lysosomal-related acidic organelles, sensitive to NAADP (nicotinic acid-adenine dinucleotide phosphate) [3,6]. Ca^{2+} is accumulated in the acidic store by the activity of SERCA3, which in contrast with SERCA2b located in the DTS, is sensitive to TBHQ. In addition, Ca^{2+} uptake into acidic organelles is driven by proton gradients maintained by vacuolar proton pumps (H^+-ATPase) [3,27,28]. We have recently reported [6], that ADP and vasopressin are unable to release Ca^{2+} from the acidic stores in human platelets; however, thrombin probably releases Ca^{2+} from the acidic organelles by the synthesis of NAADP. This conclusion is based in the inhibitory effect of nimodipine, an L-type Ca^{2+} channel inhibitor also shown to block NAADP receptors [29], on thrombin-induced release from the acidic stores and the observation that NAADP was able to release Ca^{2+} from these stores in permeabilized platelets [6].

Thrombin receptors in human platelets include the protease-activated receptors PAR-1 and PAR-4 and GPIb-IX-V, which share with the von Willebrand factor [10]. Thrombin shows a higher affinity for PAR-1 than PAR-4, and thus it is believed

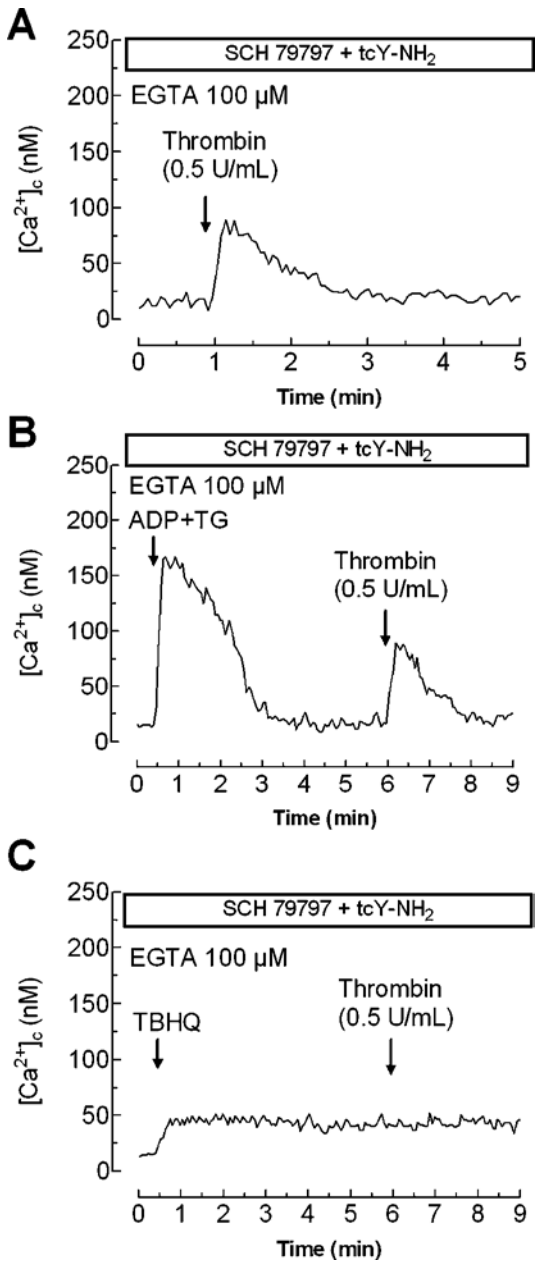

Figure 6 Effect of PAR-1 and PAR-4 antagonists on thrombin-evoked Ca^{2+} release in human platelets

Fura 2-loaded human platelets, resuspended in Ca^{2+}-free medium (100 μM EGTA was added at the time of experiment), were pre-treated with 3 μM SCH 79797 and 400 μM tcY-NH_2 for 30 min and then were stimulated with thrombin 0.5 units/ml alone (**A**), with 10 μM ADP in combination with 10 nM TG followed by the addition of 0.5 units/ml of thrombin 5.5 min later (**B**) or with 20 μM TBHQ followed by the addition of 0.5 units/ml of thrombin 5.5 min later (**C**). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of four independent experiments

that activation of human platelets by low doses of thrombin is predominantly mediated by PAR-1 [7,8]. PAR-4 has been suggested to sustain prolonged platelet activation by high concentrations of thrombin [9,30]. We have found that Ca^{2+} mobilization mediated by activation of PAR-1 is predominantly dependent on the DTS, while Ca^{2+} release in response to activation of PAR-4 depends equally on the DTS and the acidic stores.

GPIb-IX-V is a high affinity receptor for thrombin, and a body of evidence indicates that this receptor also contribute to the activation of human platelets [9,10,14–16]. We have found that Ca^{2+} mobilization mediated by activation of GPIb-IX-V by thrombin is entirely dependent on Ca^{2+} accumulated in the acidic stores and, in agreement with previous studies reporting that GPIb-IX-V binds thrombin with high affinity [10], our results indicate that thrombin completely discharges the acidic Ca^{2+} stores at concentrations as low as 0.01 units/ml and a detectable Ca^{2+} release from this store was detected when 0.001 units/ml of thrombin were used, which is expected to bind to the high affinity receptors PAR-1 and GPIb-IX-V [7–10]. Consistent with this, we found that 0.01 units/ml of thrombin released Ca^{2+} from both stores in human platelets, the DTS, which might be mostly mediated by activation of PAR-1, and the acidic stores, which are likely to be predominantly mediated by GPIb-IX-V.

Our results indicate that depletion of the acidic stores or the DTS by thrombin occurs in a concentration-dependent fashion. Ca^{2+} release from the acidic stores is mediated by the high-affinity receptors GPIb-IX-V, and to some extent PAR-1 and the low-affinity receptor PAR-4; whereas, Ca^{2+} release from the DTS is mediated by the high affinity PAR-1 and the low affinity receptor PAR-4. Thus, further platelet stimulation with higher concentrations of thrombin, which binds to high and low affinity receptors, could mediate the concentration-dependent effect of thrombin on Ca^{2+} release from the stores.

SOCE (store-operated Ca^{2+} entry), a major mechanism for Ca^{2+} entry into cells [31], is triggered by depletion of the intracellular Ca^{2+} stores. Previous studies aimed at characterizing SOCE in human platelets have reported two mechanisms for SOCE that were activated by depletion of either the DTS or the acidic stores differentially modulated by the actin cytoskeleton. The membrane-associated actin network prevents constitutive SOCE via depletion of both stores, thus reorganization of the cortical actin cytoskeleton permits the activation of Ca^{2+} entry via both mechanisms, but only SOCE activated by the depletion of the DTS requires new actin polymerization, which may support transport of portions of the DTS towards the plasma membrane [18,26,32]. As a result, inhibition of actin polymerization by Cyt D (cytochalasin D) or latrunculin A enhanced SOCE activated by the acidic stores, whereas it reduced DTS-dependent SOCE [26]. As shown previously [6], thrombin is able to release Ca^{2+} from both stores, therefore triggering both SOCE pathways simultaneously, through the activation of PAR-1, PAR-4 and GPIb-IX-V. A recent study [13] has reported that SOCE evoked by the PAR-4 agonist AYPGKF is enhanced by disruption of the actin cytoskeleton by Cyt D, whereas Ca^{2+} entry evoked by the PAR-1 agonist SFLLRN was unaffected. This phenomenon has been attributed to a direct effect of Cyt D preventing the internalization of PAR-4, which may lead to prolonged signalling from this receptor. The results presented here further support the observations of Harper and Sage [13], which showed that the PAR-4 agonist AYPGKF releases Ca^{2+} from the acidic stores more effectively than the PAR-1 agonist SFLLRN, therefore AYPGKF is a more effective activator of the acidic store-dependent SOCE pathway, the mechanism enhanced by Cyt D.

In summary, we found that thrombin induces Ca^{2+} release from the DTS and acidic compartments through the activation of PAR-1 and PAR-4 in human platelets, although the relative contribution of both stores to Ca^{2+} mobilization evoked by PAR-1 and PAR-4 receptors differs. In addition, thrombin induces activation of GPIb-IX-V, which releases Ca^{2+} solely from the acidic compartments in these cells. Identification of the intracellular Ca^{2+} stores involved in Ca^{2+} mobilization associated with the activation of thrombin receptors is a key feature for the characterization of Ca^{2+} signalling induced by this physiological agonist, which, in turn, might be essential for the investigation of

pathophysiological alterations associated with thrombin in human platelets.

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