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

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ABSTRACT

Physiological agonists increase cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) to regulate a number of cellular processes. The platelet thrombin receptors, PAR-1, PAR-4 and GPIb-IX-V have been described as potential contributors of thrombin-induced platelet aggregation. Platelets present two separate Ca^{2+} stores, the dense tubular system (DTS) and acidic organelles, differentiated by the distinct sensitivity of their respective SERCAs to thapsigargin and 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ). 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. Desensitisation 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+} mobilisation induced by thrombin, and depletion of the DTS after desensitisation 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 reported 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 desensitised 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.

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INTRODUCTION

Human platelets possess two separate agonist-releasable Ca^{2+} stores differentiated by the distinct sensitivity of the SERCA isoforms located on each store to thapsigargin (TG) and 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ). The major store has long been identified as the dense tubular system (DTS). 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-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, respectively, are potent and selective activators of PAR-1 and PAR-4 and trigger thrombin-evoked platelet responses [8, 11-13].

GPIb-IX-V binds thrombin with high affinity and may contribute to the activation of platelets [14, 15]. Platelet activation by low concentrations of thrombin has been shown to be 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 desensitisation activates a number of intracellular pathways, including phosphorylation of MAPK 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+} mobilisation 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.

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MATERIAL AND METHODS

Materials

Fura-2 acetoxymethyl ester (fura-2/AM), and calcein were from Molecular Probes (Leiden, The Netherlands). Apyrase (grade V), aspirin, thrombin, ADP, bovine serum albumin (BSA), ionomycin (Iono) and TG were from Sigma (Madrid, Spain). SFLLRN and AYPGKF were from Bachem (Merseyside, UK). TcY-NH_2 and SCH 79797 were from Tocris (Bristol, UK). TBHQ was from Alexis (Nottingham, UK). 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 anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid and 111 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 °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 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.

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-AM at 37 ºC, centrifuged 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. 95 % of cells were viable after treatment with the inhibitors, similar to that observed in our resting platelet suspensions.

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

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 and coworkers [19]. Thrombin, TG+Iono, SFLLRN or AYPGKF-induced Ca^{2+} release was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 2 ½ min after their addition taking a sample every second and 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.

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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 U/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 in $[Ca^{2+}]_c$ (Figure 1A), which, as expected, was found to be concentration dependent (Figure 1B). Treatment of platelets with TBHQ (20 μM), 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 U/mL to a similar extent (Figure 1B), which suggests that at concentrations as low as 0.01 U/mL thrombin discharges the acidic stores in platelets. To confirm this issue we have performed a series of experiments that reported that treatment of platelets with thrombin (0.01 U/mL) alone or in combination with 20 μM TBHQ 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 on $[Ca^{2+}]_c$ to basal levels after cell stimulation was slower in the presence of TBHQ, which is likely due to the inhibition of SERCA3, and therefore the impairment of the reuptake of calcium into the TBHQ-sensitive store. In addition, we have investigated whether pre-treatment with thrombin (0.01 U/mL) prevents a 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 U/mL thrombin abolished TBHQ-induced calcium 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, which is present on the membrane of the DTS and shows high sensitivity to TG [2, 21, 22], in combination with ADP (10 μM), which selectively releases Ca^{2+} from the DTS without affecting the acidic stores [6] and is required to induce rapid discharge of

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the store. In a Ca^{2+}-free medium platelet stimulation with ADP did not alter subsequent Ca^{2+}-release induced by thrombin (0.5 U/mL), suggesting that the use of ADP did not desensitise the thrombin receptors (the integral of the rise in $[Ca^{2+}]_c$ for 2 ½ min after the addition of thrombin was 16433 ± 478 and 16215 ± 341 nM·s in the absence or presence of ADP, Figure 2A and D; $P>0.05$; n=6). In addition, treatment with ADP did not significantly reduce Ca^{2+}-release induced by addition of TG (1 μM) in combination with Iono (50 nM) to fully deplete the intracellular Ca^{2+} stores (the integral of the rise in $[Ca^{2+}]_c$ for 2 ½ min after the addition of TG+Iono was 20226 ± 784 and 19261 ± 657 nM·s in the absence and presence of ADP), which indicates that Ca^{2+} release induced by ADP is rapidly reuptaked into the DTS and support the lack of effect of ADP on thrombin-evoked response (Figure 2B and D; $P>0.05$; n=6).

In order to investigate whether the treatment with ADP + TG is effectively inducing depletion of the DTS, we tested the effect of thrombin (0.5 U/mL) in platelets where the DTS had been discharged by treatment with ADP + TG, followed by depletion of the acidic stores by addition of TBHQ (20 μM). As depicted in Figure 2C and 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, pre-treated with ADP (10 μM) + TG (10 nM) to deplete the DTS, with thrombin, at concentrations 0.001 to 0.5 U/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 ½ min after the addition of thrombin 0.001, 0.005, 0.01 and 0.5 U/mL was 920 ± 200, 3693 ± 996, 6427 ± 764 and 6500 ± 850 nM·s, respectively; Figure 2E; n=4).

As shown in Figure 3A, 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 1 mM for AYPGKF and an EC_{50} of 1.4 and 27 μM, respectively (Figures 3C and D; 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 than the maximal concentrations of

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the agonists (Figure 3C and D), and have been previously used by others (23, 24).

Ca^{2+} release evoked by SFLLRN (10 μM) was significantly reduced by 80 ± 10 % when the DTS had previously been depleted by treatment with 10 μM ADP + 10 nM TG (the integral of the rise in $[Ca^{2+}]_c$ for 2 ½ min after the addition of SFLLRN taking a sample every second 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 + TG reduced Ca^{2+} release evoked by AYPGKF (500 μM) by only 50 ± 7 % (the integral of the rise in $[Ca^{2+}]_c$ for 2 ½ 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 on the DTS

Characterisation of Ca^{2+} mobilization upon activation of the GPIb-IX-V receptor by thrombin was investigated by desensitising PAR-1 and PAR-4 receptors. Desensitisation was achieved by platelet treatment with SFLLRN (10 μM) in combination with AYPGKF (750 μM). As shown in Figure 4A, desensitisation of PAR-1 and PAR-4 was confirmed by the inability of SFLLRN and AYPGKF to increase $[Ca^{2+}]_c$ in PAR-desensitised platelets. Subsequent addition of TG (1 μM) in combination with Iono (50 nM) 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 ½ min after the addition of TG+Iono was 19098 ± 906 nM·s, similar to that found in control cells, see Figure 2D; n=6). Preliminary experiments reported that PAR-4 desensitisation was not completely achieved by AYPGKF at 500 μM and a number of platelet preparations still responded slightly to second addition of the peptide; however, no response was observed when AYPGKF was used at 750 μM.

Platelet stimulation with thrombin (0.5 U/mL) when PAR-1 and PAR-4 receptors had been desensitised 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 ½ min after the addition of thrombin was 16730 ± 1430 and 3462 ± 459 nM.s in control and PAR-desensitised platelets, respectively. In cells where PAR-1 and PAR-4 had been desensitised, depletion of the DTS using ADP + TG had no significant effect on thrombin-evoked Ca^{2+} release (the integral of the rise in $[Ca^{2+}]_c$ for 2 ½ min after the addition of thrombin was 3398 ± 406 nM.s in PAR-desensitised cells treated with ADP+TG; Figure 4C; $P>0.05$; n=6).

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

As previously shown [3, 6] and depicted in Figure 2C, treatment of platelets with 20 μM TBHQ fully depletes the acidic compartments. In a 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 ½ min after the addition of SFLLRN was 11786 ± 1293 and 9402 ± 2031 nM.s in the absence or presence of TBHQ; Figure 5A; n=6; $P<0.05$). 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 ½ 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 of Ca^{2+} mobilization from the DTS.

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 desensitised 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 than in cells where PAR-1 and PAR-4 were operative (see Figure 5C vs. 1A). The reduction in thrombin-evoked Ca^{2+} mobilization was not due to store depletion, since treatment with TG (1 μM) + Iono (50 nM), 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 desensitised, 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 ½ min after the addition of thrombin was 3462 ± 459 and 684 ± 35 nM.s in PAR-desensitised cells in the absence or presence of TBHQ; Figure 5D vs. 5C; n=6; $P<0.01$).

These findings were confirmed by 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 have 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 (data not shown). As shown in Figure 6A, in cells pre-treated with the PAR antagonists thrombin-evoked Ca^{2+} release was significantly reduced as reported by PAR desensitisation (the integral of the rise in $[Ca^{2+}]_c$ for 2 ½ 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 + TG (the integral of the rise in $[Ca^{2+}]_c$ for 2 ½ 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

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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 IP_3 generation [6]. The second store has been identified as lysosomal-related acidic organelles, sensitive to NAADP [3, 6]. Ca^{2+} is accumulated in the acidic store by the activity of SERCA3, which in contrast to 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 that ADP and vasopressin are unable to release Ca^{2+} from the acidic stores in human platelets; however, thrombin releases Ca^{2+} from the acidic organelles probably by the synthesis of NAADP [6]. 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 the glycoprotein GPIb-IX-V, which shares with the von Willebrand factor [10]. Thrombin shows a higher affinity for PAR-1 than PAR-4, and thus it is believed 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+} mobilisation mediated by activation of PAR-1 is predominantly dependent on the DTS, while Ca^{2+} release in response to activation of PAR-4 equally depends on the DTS and the acidic stores.

GPIb-IX-V is a high affinity receptor for thrombin, and a body of evidence indicate that this receptor also contribute to the activation of human platelets [9, 10, 14-16]. We have found that Ca^{2+} mobilisation 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 stores at concentrations as low as 0.01 U/mL and a detectable Ca^{2+} release

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from this stores was detected at 0.001 U/mL thrombin, which is expected to bind to the high affinity receptors PAR-1 and GPIb-IX-V [7-10]. Consistent with this, we found that thrombin 0.01 U/mL releases 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 is likely to be predominantly mediated by the 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, while 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, would mediate the concentration-dependent effect of thrombin on Ca^{2+} release from the stores.

Store-operated Ca^{2+} entry (SOCE), a major mechanism for Ca^{2+} entry into cells [31], is triggered by depletion of the intracellular Ca^{2+} stores. Previous studies aimed to characterise SOCE in human platelets have reported two mechanisms for SOCE 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, reorganisation 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 polymerisation, which may support transport of portions of the DTS towards the plasma membrane [26, 32, 33]. As a result inhibition of actin polymerisation by cytochalasin D (Cyt D) or latrunculin A enhanced SOCE activated by the acidic stores whereas reduced DTS-dependent SOCE [26]. As previously mentioned [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. Recent studies have 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 internalisation of PAR-4, which may lead to prolonged signalling from this receptor [13]. Our results further support this report considering that PAR-4 agonist, AYPGKF, releases Ca^{2+} from the acidic stores more effectively than 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

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compartments through the activation of PAR-1 and PAR-4 in human platelets, although the relative contribution of both stores to Ca^{2+} mobilisation 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+} mobilisation associated to the activation of thrombin receptors is a key feature for the characterisation of Ca^{2+} signalling induced by this physiological agonist, which, in turn, might be essential for the investigation of pathophysiological alterations associated to thrombin in human platelets.

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Abbreviations used: $[Ca^{2+}]_c$, cytosolic free calcium concentration; Cyt D, cytochalasin D; DTS, dense tubular system; HBS, HEPES-buffered saline; Iono; ionomycin; TG, thapsigargin; TBHQ, 2,5-di-(tert-butyl)-1,4-hydroquinone; SOCE, store-operated calcium entry.

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FIGURE LEGENDS

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

(**A**) Fura 2-loaded human platelets were treated in a Ca^{2+}-free medium (100 μM EGTA was added) for 9 min at 37 ºC with 20 μM TBHQ (bold trace) or the vehicle (DMSO, plain trace). Cells were then stimulated with thrombin (0.5 U/mL). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of 6 independent experiments. (**B**) Data represent Ca^{2+} release induced by increasing concentrations of thrombin in the absence (open squares) o presence (solid circles) of TBHQ. Values are means ± S.E.M. Dashed line represents the difference between both curves. (**C**) Fura 2-loaded human platelets were stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) at 37 ºC with 0.01 U/mL thrombin alone (*trace* a) or in combination with 20 μM TBHQ (*trace b*) or were stimulated with 0.01 U/mL thrombin and 30 s later TBHQ was added as indicated by the thick arrow. Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of 4 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, suspended in a Ca^{2+}-free medium (100 μM EGTA was added at the time of experiment), were treated with ADP (10 μM, bold traces) or the vehicle (HBS, plain traces) and four min later thrombin (0.5 U/mL; **A**) or TG (1 μM) + Iono (50 nM; **B**) were added to release Ca^{2+} from the stores. (**C**) Fura 2-loaded human platelets were treated in a Ca^{2+}-free medium with ADP (10 μM) in combination with TG (10 nM) to deplete the DTS, and four min later TBHQ (20 μM) was added to discharge the acidic compartments. Five min later cells were stimulated with 0.5 U/mL thrombin. Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of 6 independent experiments. (**D**) Histograms represent Ca^{2+} release stimulated by thrombin or TG+Iono under different experimental conditions as indicated. Values are means ± S.E.M. $*P<0.01$. (**E**) Fura 2-loaded human platelets were treated in a Ca^{2+}-free medium with ADP (10 μM) in combination with TG (10 nM) to deplete the DTS, and four min later cells were stimulated with thrombin (0.5, 0.01, 0.005 and 0.001 U/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 4 independent experiments.

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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, suspended in a Ca^{2+}-free medium (100 μM EGTA was added at the time of experiment), were treated with ADP (10 μM) in combination with TG (10 nM, bold traces) or the vehicle (HBS, plain traces) and four min later SFLLRN (10 μM; **A**) or AYPGKF (500 μM; **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 6 to 8 independent experiments. (**C** and **D**) Fura 2-loaded human platelets, suspended in a 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. Values are means ± S.E.M. of 4 independent experiments.

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 a Ca^{2+}-free medium (100 μM EGTA was added at the time of experiment) with SFLLRN (10 μM) in combination with AYPGKF (750 μM) to desensitise PAR-1 and PAR-4. Cells were treated 9 ½ min later with SFLLRN (10 μM) followed by addition of AYPGKF (500 μM) 1 ½ min later. TG (1 μM) + 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 a Ca^{2+}-free medium with SFLLRN (10 μM) in combination with AYPGKF (750 μM) to desensitise PAR-1 and PAR-4. Cells were then either stimulated with thrombin (0.5 U/mL; **B**) or with ADP (10 μM) in combination with TG (10 nM) to deplete the DTS, followed by addition of thrombin (0.5 U/mL) five min later (**C**). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of 6 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, suspended in a Ca^{2+}-free medium (100 μM EGTA was added at the time of experiment), were treated with TBHQ (20 μM, bold traces) or the vehicle (DMSO, plain traces) and nine min later SFLLRN (10 μM; **A**) or AYPGKF (500 μM; **B**) were added to release Ca^{2+} from the stores. (**C** and **D**) Fura 2-loaded human platelets were

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treated in a Ca^{2+}-free medium with SFLLRN (10 μM) in combination with AYPGKF (750 μM) to desensitise PAR-1 and PAR-4. Eleven min later cells were either stimulated with thrombin (0.5 U/mL) followed by addition of TG (1 μM) + Iono (50 nM; **C**) or were treated with TBHQ (20 μM) to discharge the acidic compartments followed by addition of thrombin (0.5 U/mL). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of 6 independent experiments.

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

Fura 2-loaded human platelets, suspended in a 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 U/mL alone (**A**), with ADP (10 μM) in combination with TG (10 nM) followed by the addition of thrombin (0.5 U/mL) 5½ min later (**B**) or with TBHQ (20 μM) followed by the addition of thrombin (0.5 U/mL) 5½ min later (**C**). Changes in $[Ca^{2+}]_c$ were monitored as described in the Materials and methods section. Traces are representative of 4 independent experiments.

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Figure 1

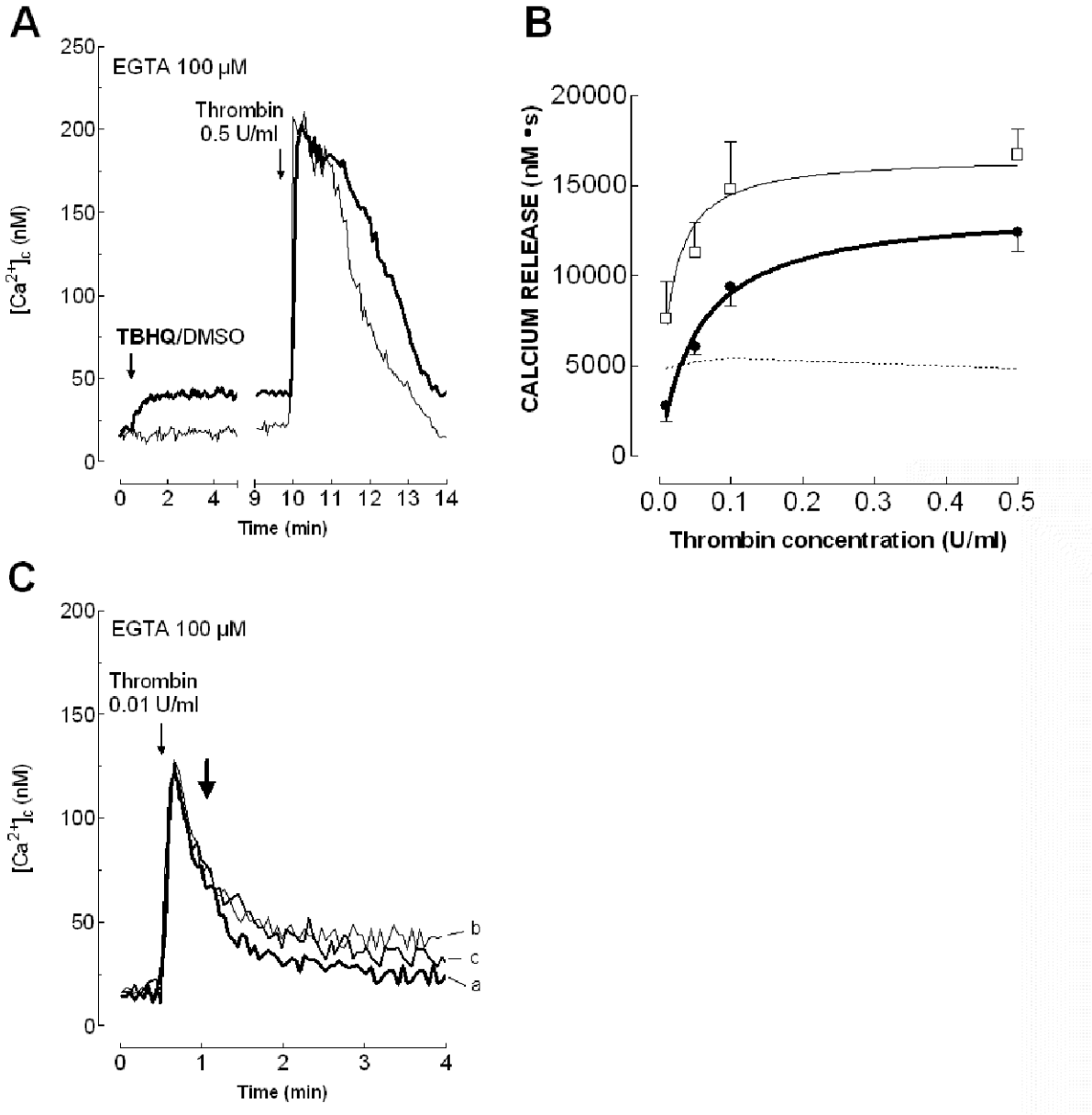

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Figure 2

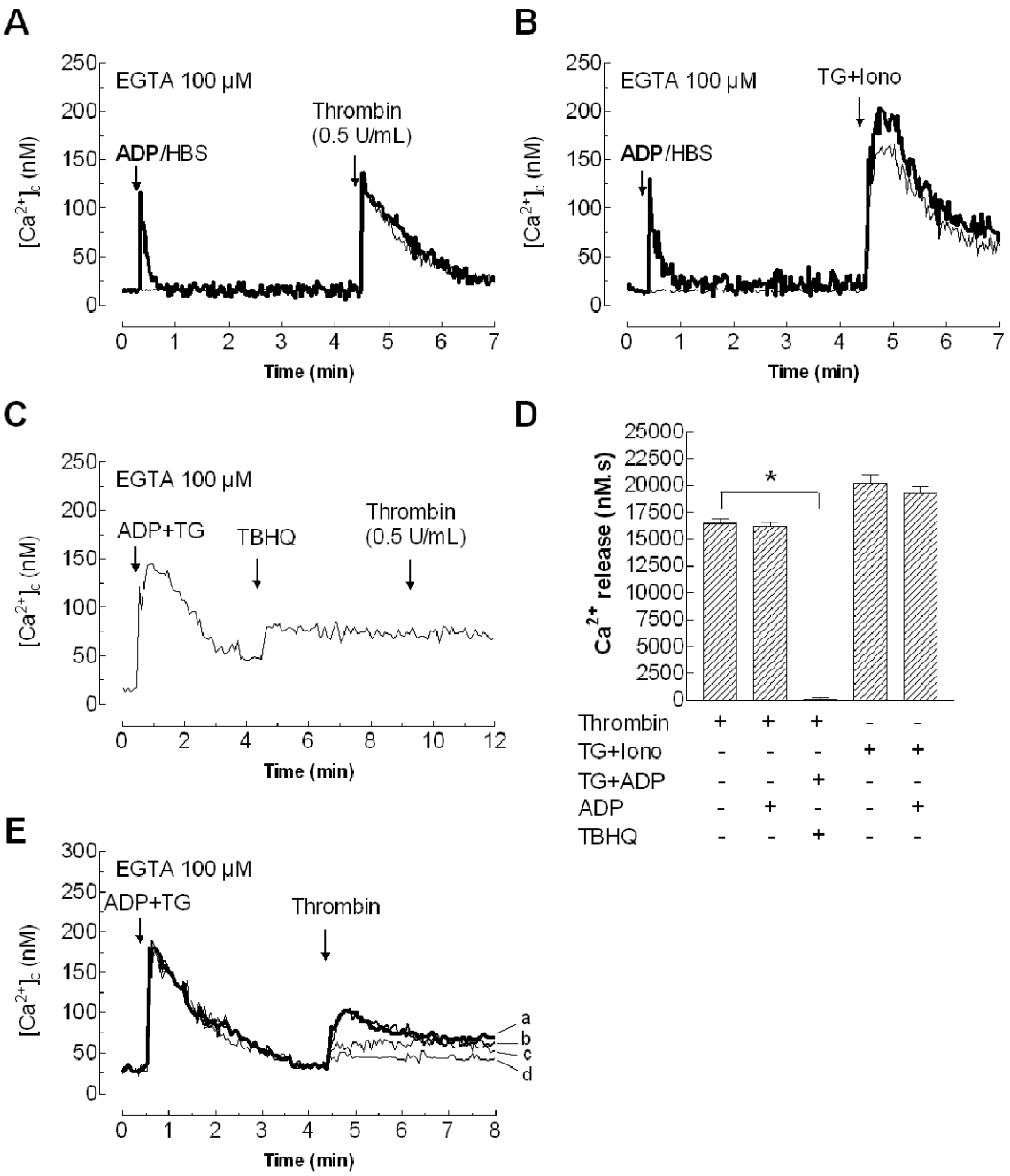

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Figure 3

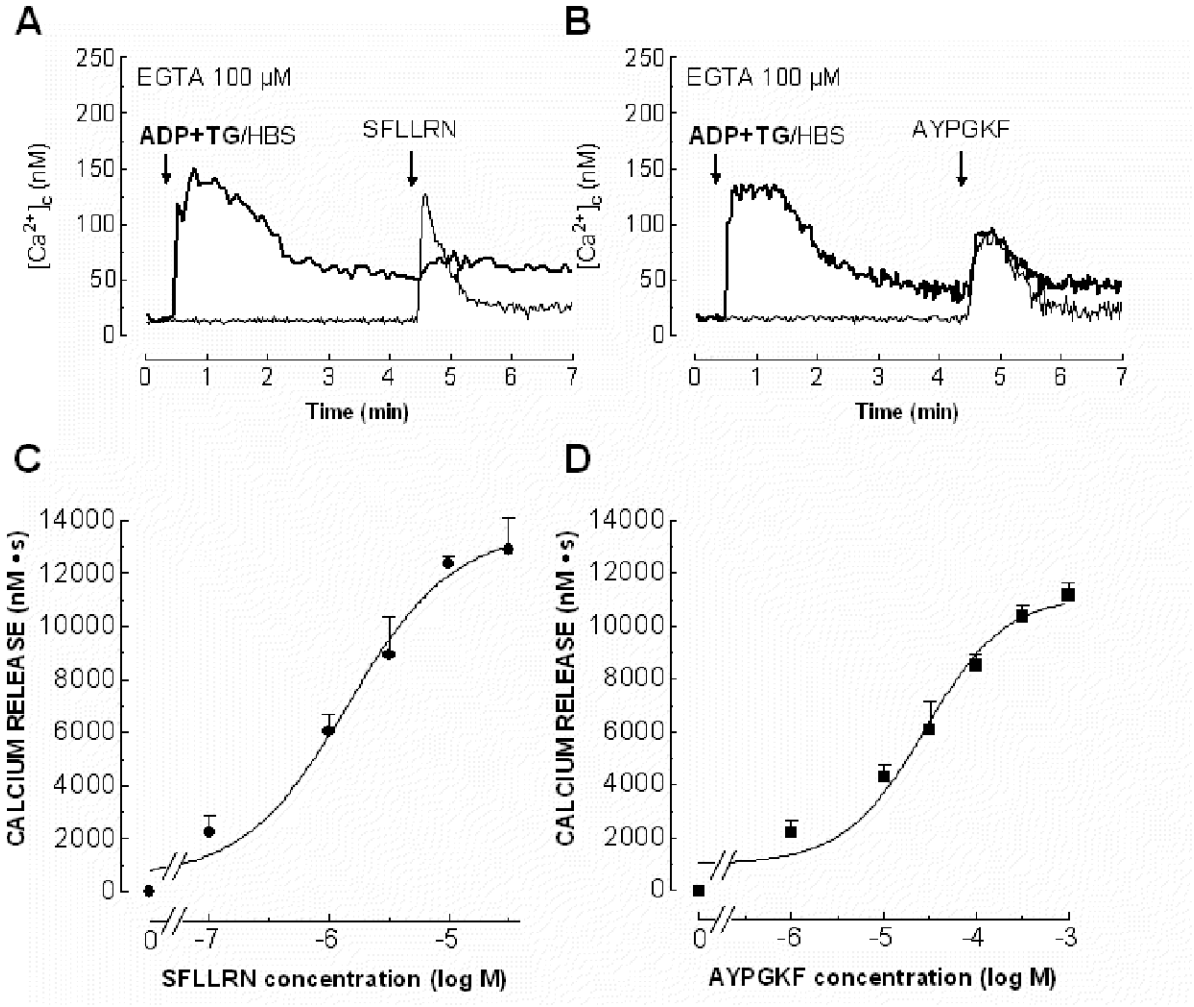

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Figure 4

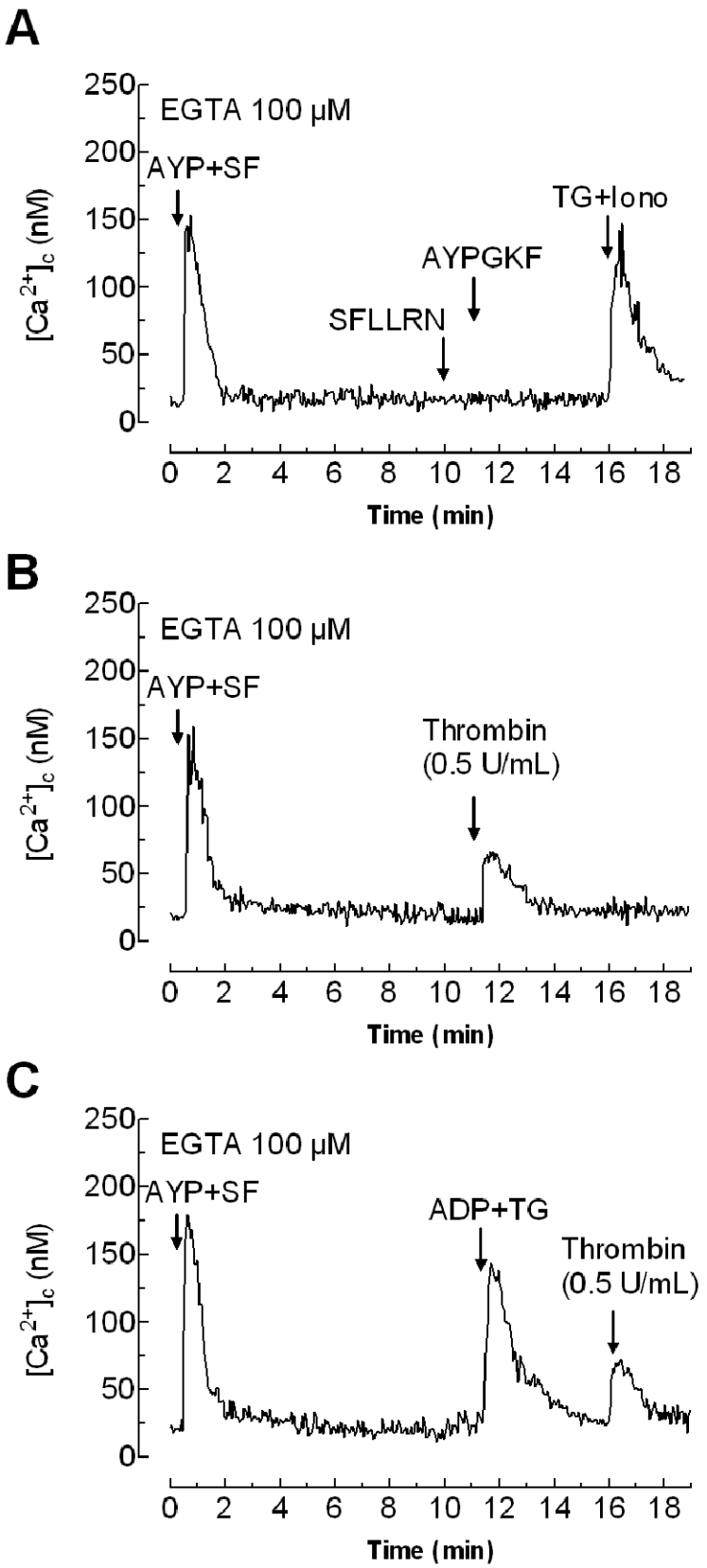

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Figure 5

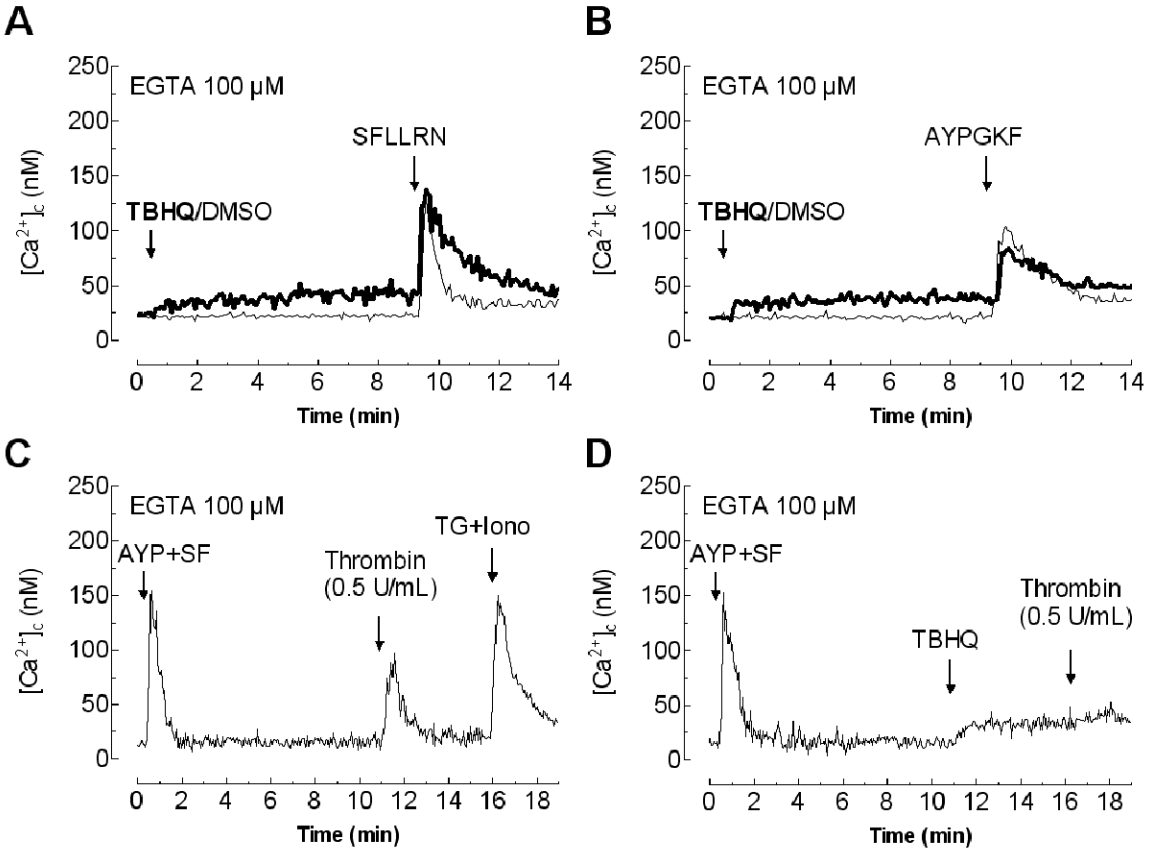

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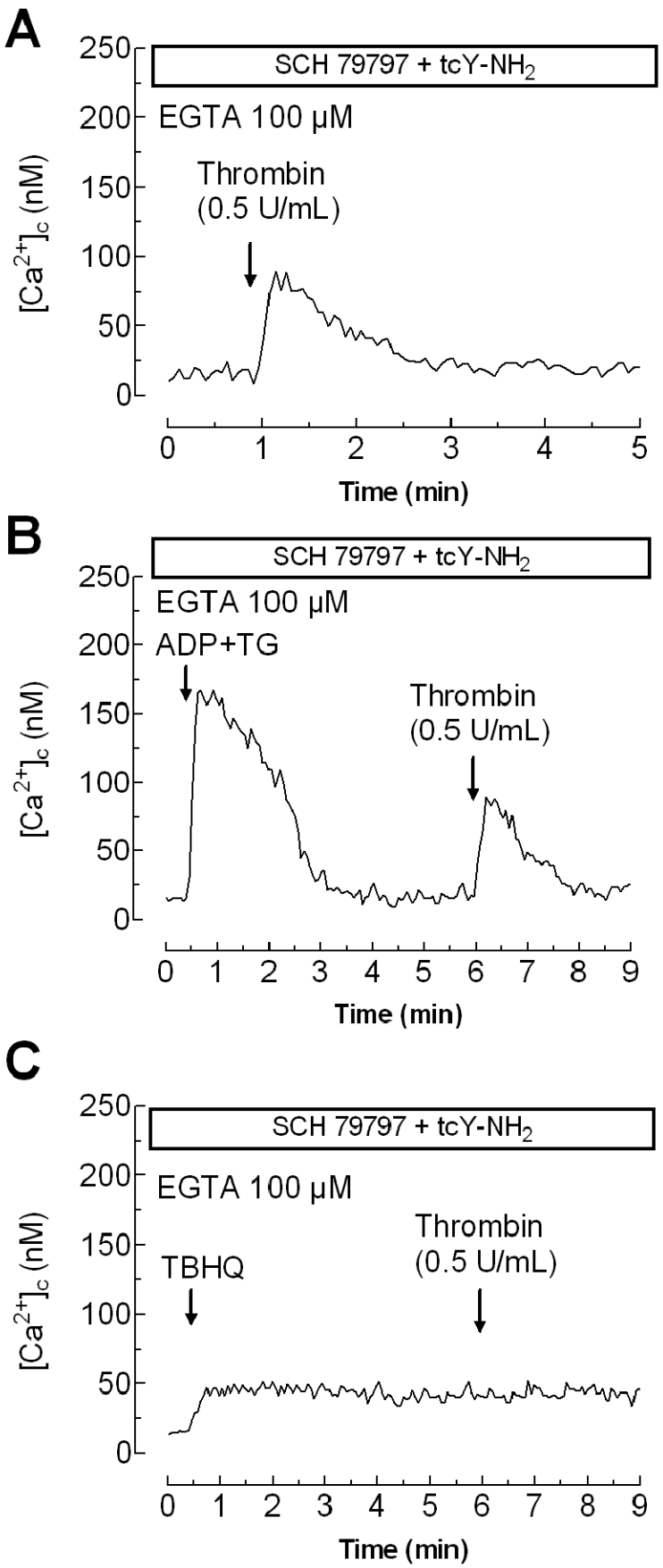
Figure 6



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