Acidic-store depletion is required for human platelet aggregation

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Platelet stimulation with thrombin induces an elevation in cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_c$) due to Ca^{2+} release from intracellular stores and entry from the extracellular medium. Two different intracellular Ca^{2+} stores have been described in human platelets: the dense tubular system and the lysosomal-like acidic stores. In the present study, we investigated the contribution of the acidic stores in thrombin-induced platelet aggregation. We have found that platelet aggregation induced by thrombin is reduced in a Ca^{2+}-free medium. Discharge of the acidic Ca^{2+} stores by treatment with the sarcoendoplasmic Ca^{2+}-ATPase (SERCA)3 selective inhibitor 2,5-di-(tert-butyl)-1,4-hydroquinone reduced thrombin-evoked platelet aggregation. In the presence of 2,5-di-(tert-butyl)-1,4-hydroquinone, platelet aggregation induced by the protease-activated receptor (PAR)-1 and PAR-4 agonist peptides, SFLLRN and AYPGKF, respectively, was significantly reduced. In cells with depleted acidic stores, activation of GPIb-IX-V by thrombin resulted in reduced or no platelet aggregation in a medium containing 1 mmol/l Ca^{2+}or in a Ca^{2+}-free medium, respectively. This finding suggests that Ca^{2+} accumulation in the acidic Ca^{2+} compartments is required for platelet aggregation induced by activation of the G-coupled PAR-1 and PAR-4 thrombin receptors and, by the occupation of the leucine-rich glycoprotein GPIb-IX-V and provide evidence supporting a functional role of the lysosomal-like acidic Ca^{2+} stores in human platelets. *Blood Coagul Fibrinolysis* 20:511–516
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Blood Coagulation and Fibrinolysis 2009, **20**:511–516

Keywords: aggregation, AYPGKF, platelets, SFLLRN, TBHQ and acidic stores, thrombin

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Received 12 June 2008 **Revised** 3 October 2008
Accepted 11 November 2008

Introduction

Cytosolic Ca^{2+} is a focal point of many signal transduction pathways and regulates cellular activities ranging from motility to cell death [1]. The endoplasmic reticulum or its analogue in platelets, the dense tubular system (DTS), represents the main Ca^{2+} store in most cell types. In addition, several other cellular organelles also store Ca^{2+} and act as physiological agonist-releasable Ca^{2+} compartments. Among the organelles that accumulate Ca^{2+} are mitochondria, nuclear envelope, Golgi apparatus, secretory granules and lysosomes [2–8]. Like other cells, platelets have a complex mechanism for Ca^{2+} signalling in which the sarcoendoplasmic Ca^{2+}-ATPase (SERCA) plays a determinant role. In these cells, two different SERCA isoforms have been shown to be distributed separately in two distinct Ca^{2+} stores: SERCA2b and SERCA3 [9–11]. Each isoform shows a distinct sensitivity to thapsigargin and 2,5-di-(tert-butyl)-1,4-hydroquinone (TBHQ) [10–12]. Recent works provide evidences that the TBHQ-sensitive Ca^{2+} store in human platelets is mainly a lysosomal store [13].

Platelets play a critical role in thrombosis and haemostasis. These cells are activated by various soluble agonists, including thrombin and ADP, and adhesive proteins, such as collagen and von Willebrand factor, via different signalling pathways. Most agonists mobilize Ca^{2+} or are modulators of Ca^{2+} homeostasis. Thrombin exerts its cellular effects mainly through the activation of the protease-activated receptors (PARs): PAR-1 and PAR-4 [14–16], an effect that can be mimicked by the PAR-1 and PAR-4 agonist peptides SFLLRN and AYPGKF, respectively, and the leucine-rich glycoprotein GPIb-IX-V [17,18].

The events associated to platelet activation by thrombin include activation of membrane receptors, shape change, granular secretion, cytoskeletal remodelling, and aggregation. Changes in shape, and aggregation that accompanies platelet activation, are dependent on the assembly and reorganization of the cytoskeleton, which has been reported to be necessary for the irreversible phase of platelet aggregation [19]. In previous studies we reported that treatment of platelets with SFLLRN induces a time-dependent and biphasic increase in actin filament polymerization; similar results were observed when platelets were stimulated with the PAR-4 agonist AYPGKF [20].

0957-5235 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI:10.1097/MBC.0b013e32832f5104

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Cytosolic Ca^{2+} mobilization has been reported to be essential for thrombin-induced full platelet activation; however, the involvement of the acidic Ca^{2+} stores in thrombin-evoked platelet aggregation remains unclear. Here we have investigated the contribution of the TBHQ-sensitive acidic organelles in platelet aggregation induced by stimulation of the thrombin receptors PAR-1, PAR-4 and the leucine-rich glycoprotein GPIb-IX-V.

Materials and methods

Materials

Apyrase (grade VII), ethylene glycol tetraacetic acid (EGTA), aspirin, bovine serum albumin (BSA), thrombin, were from Sigma (Madrid, Spain). SFLLRN and AYPGKF were from Bachem (Merseyside, UK). TBHQ was from Alexis (Nottingham, UK). Calcein was from Molecular Probes (Leiden, The Netherlands). Anti-GPIb monoclonal antibody AK2 was from Serotec Ltd. (Kidlington, UK). All other reagents were purchased from Panreac (Barcelona, Spain). The thrombin preparation (specific activity P2000 NIH units/mg protein) was predominantly α-thrombin, containing minimum autolytic digestion products, according to the manufacturer's instructions; therefore, most of the effects shown in the present study should be attributed to α-thrombin.

Platelet preparation

Blood was obtained from healthy volunteers as approved by local ethical committees and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mmol/l): 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 μmol/l) and apyrase (40 μg/ml) added. Cells were then collected by centrifugation at 350*g* for 20 min and resuspended in Hepes-buffered saline (HBS) containing (in mmol/l): 145 NaCl, 10 Hepes, 10 D-glucose, 5 KCl, 1 $MgSO_4$ (pH 7.45), and supplemented with 0.1% w/v BSA.

Cell viability

Cell viability was assessed using calcein and trypan blue. For calcein loading, cells were incubated for 30 min with 5 μmol/l calcein-AM at 37°C, centrifuged and the pellet was resuspended in fresh HBS. Fluorescence was recorded from 2-ml aliquots using a spectrophotometer (Varian Ltd., Madrid, Spain). Samples were excited at 494 nm and the resulting fluorescence was measured at 535 nm. The results obtained with calcein were confirmed using the trypan blue exclusion technique. Ninety-five percent of cells were viable in our platelet suspensions.

Platelet aggregation

The percentage, rate and lag-time of aggregation in washed platelets were monitored using a Chronolog (Havertown, PA, USA) aggregometer at 37°C under stirring at 1200 r.p.m. [20]. The percentage of aggregation or amplitude is estimated as the percentage of the difference in light transmission between the platelet suspension in HBS and HBS alone and indicates the percentage of platelets that aggregate in response to an agonist. Resting platelets in suspension are arbitrarily considered by the aggregometer as 0% aggregation and HBS is considered to be 100% aggregation. The rate, or slope, of the aggregation is the percentage change of aggregation per minute.

Statistical analysis

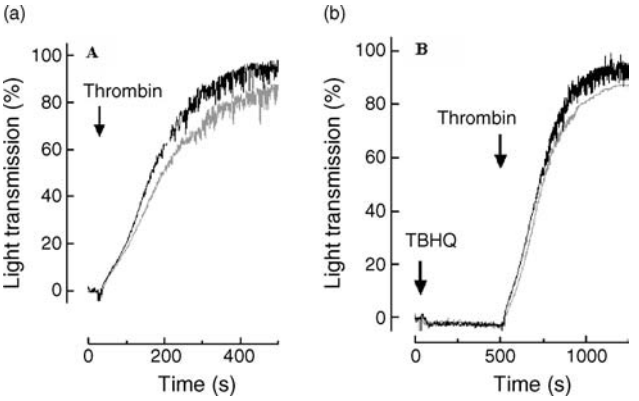
Analysis of statistical significance was performed using Student's *t*-test. $P < 0.05$ was considered to be significant for a difference.

Results

Treatment of human platelets in the presence of 1 mmol/l extracellular Ca^{2+} with 1 U/ml thrombin induces rapid aggregation indicated by a large increase in light transmission. This effect of thrombin on platelet aggregation was significantly reduced in a Ca^{2+}-free medium (100 μmol/l EGTA was added to the medium; Fig. 1a and Table 1; $P < 0.05$ Student's *t*-test, $n = 10$). Platelet stimulation with 1 U/ml thrombin once the acidic stores have been discharged using the SERCA3 inhibitor TBHQ (20 μmol/l) significantly reduced platelet aggregation as compared with the experiment in the absence of TBHQ (Fig. 1b and Table 1; $P < 0.05$ Student's *t*-test, $n = 10$).

We have further investigated the contribution of the acidic stores in platelet aggregation induced by activation of the thrombin receptors PAR-1, PAR-4 and the leucine-rich glycoprotein receptor GPIb-IX-V by using SFLLRN

Fig. 1



Platelet aggregation by activation of thrombin receptors. Human platelets were suspended in HBS containing 1 mmol/l Ca^{2+} (black traces) or in a Ca^{2+}-free HBS (grey traces) and then stimulated with 1 U/ml thrombin (a), or preincubated with 20 μmol/l TBHQ for 8 min and then stimulated with 1 U/ml (b). Aggregation of human platelets was induced at a shear rate of 1200 r.p.m. at 37°C in an aggregometer as described in Materials and methods section. Traces shown are representative of 6–10 separate experiments. HBS, Hepes-buffered saline; TBHQ, 2,5-di-(tert-butyl)-1,4-hydroquinone.

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Table 1 Platelet aggregation by occupation of thrombin receptors

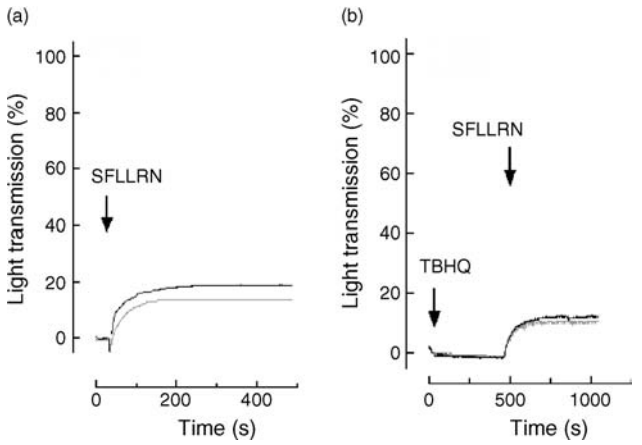
Stimulatory agent	Lag-time (min)	% Rate	% Aggregation
1 mmol/l extracellular Ca^{2+}			
Thrombin 1 U/ml	0.02 ± 0.01	57 ± 4	92 ± 1
TBHQ/thrombin	$0.08 \pm 0.04^{*}$	$50 \pm 4^{*}$	$87 \pm 2^{*}$
SFLLRN 10 μmol/l	0.02 ± 0.01	31 ± 2	16 ± 1
TBHQ/SFLLRN	$0.07 \pm 0.03^{*}$	35 ± 6	$12 \pm 1^{*}$
AYPGKF 500 μmol/l	0.19 ± 0.05	35 ± 2	54 ± 2
TBHQ/AYPGKF	$1.20 \pm 0.10^{*}$	$20 \pm 6^{*}$	$35 \pm 6^{*}$
[PARs-desensitized cells] thrombin 1 U/ml	1.05 ± 0.01	46 ± 8	52 ± 2
TBHQ/PAR-desensitized thrombin	1.10 ± 0.1	46 ± 5	$47 \pm 2^{*}$
Ca^{2+}-free medium			
Thrombin 1 U/ml	$0.05 \pm 0.01^{**}$	58 ± 6	$86 \pm 1^{**}$
TBHQ/thrombin	0.07 ± 0.01	47 ± 5	87 ± 2
SFLLRN 10 μmol/l	$0.07 \pm 0.01^{**}$	$18 \pm 3^{**}$	$12 \pm 1^{**}$
TBHQ/SFLLRN	0.06 ± 0.03	$18 \pm 3^{**}$	11 ± 1
AYPGKF 500 μmol/l	0.19 ± 0.03	$18 \pm 3^{**}$	$18 \pm 3^{**}$
TBHQ/AYPGKF	$1.23 \pm 0.05^{*}$	$10 \pm 4^{*,**}$	$6 \pm 1^{*,**}$
[PARs-desensitized cells] thrombin 1 U/ml	$2.15 \pm 0.04^{**}$	46 ± 4	$41 \pm 2^{**}$
TBHQ/PAR-desensitized thrombin	$2.80 \pm 0.10^{*,**}$	$15 \pm 3^{*,**}$	$8 \pm 1^{*,**}$

HBS, Hepes-buffered saline; PAR, protease-activated receptor; TBHQ, 2,5-di-(tert-butyl)-1,4-hydroquinone. Human platelets were suspended in a HBS containing 1 mmol/l Ca^{2+} or in a Ca^{2+}-free HBS and pretreated in the absence of presence of 20 μmol/l TBHQ for 8 min. Cells were then stimulated with 1 U/ml thrombin, 10 μmol/l SFLLRN, or 500 μmol/l AYPGKF. In addition, platelets have been treated with SFLLRN (10 μmol/l) in combination with AYPGKF (500 μmol/l) to desensitize PAR-1 and PAR-4 and 10 min later were stimulated with thrombin 1 U/ml. Platelet aggregation was determined as described under Material and methods section. Values given are presented as mean ± SEM of 6–10 separate determinations. $^{*}P < 0.05$ compared to the responses in the absence of TBHQ pretreatment. $^{**}P < 0.05$ compared to the responses in the presence of 1 mmol/l extracellular Ca^{2+}.

and AYPGKF at concentrations that cause maximal cell effects, 20 and 500 μmol/l, respectively [21]. As shown in Fig. 2a and Table 1, treatment of platelets with 20 μmol/l SFLLRN, induced only the reversible phase without given full aggregation, in agreement with previous studies [20,22,23]. As previously reported for thrombin, the SFLLRN-induced aggregation rate and amplitude was reduced in absence of extracellular Ca^{2+} (Fig. 2a and Table 1). Discharge of the acidic stores with TBHQ prior to the stimulation with SFLLRN reduced platelet aggregation induced by PAR-1 activation in the presence of 1 mmol/l extracellular Ca^{2+} (Fig. 2a and Table 1; $P < 0.05$ Student's *t*-test, $n = 6$), although no significant effect was observed in the absence of extracellular Ca^{2+}. As a result, in the presence of 20 μmol/l TBHQ SFLLRN-induced aggregation was similar in the absence and presence of extracellular Ca^{2+} (Fig. 2b and Table 1).

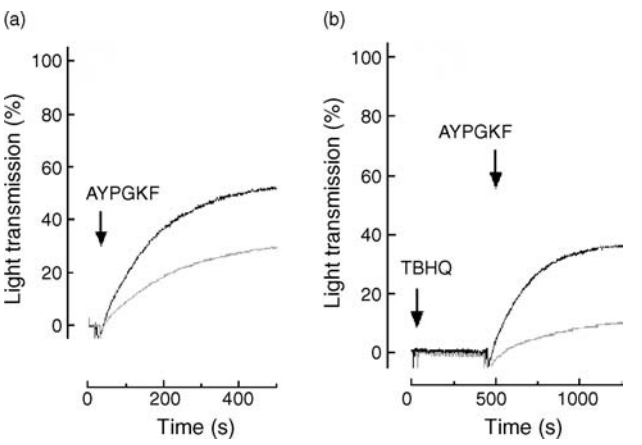
Stimulation of platelets with 500 μmol/l AYPGKF in a medium containing 1 mmol/l Ca^{2+} induced platelet

Fig. 2



Platelet aggregation by activation of PAR-1. Human platelets were suspended in HBS containing 1 mmol/l Ca^{2+} (black traces) or in a Ca^{2+}-free HBS (grey traces) and then stimulated with 10 μmol/l SFLLRN (a), or preincubated with 20 μmol/l TBHQ for 8 min and then stimulated with 10 μmol/l SFLLRN (b). Aggregation of human platelets was induced at a shear rate of 1200 r.p.m. at 37°C in an aggregometer as described in Materials and methods section. Traces shown are representative of six separate experiments. HBS, Hepes-buffered saline; PAR, protease-activated receptor; TBHQ, 2,5-di-(tert-butyl)-1,4-hydroquinone.

Fig. 3



Platelet aggregation by activation of PAR-4. Human platelets were suspended in HBS containing 1 mmol/l Ca^{2+} (black traces) or in a Ca^{2+}-free HBS (grey traces) and then stimulated with 500 μmol/l AYPGKF (a), or preincubated with 20 μmol/l TBHQ and then stimulated with 500 μmol/l AYPGKF (b). Aggregation of human platelets was induced at a shear rate of 1200 r.p.m. at 37°C in an aggregometer as described in Materials and methods section. Traces shown are representative of six separate experiments. HBS, Hepes-buffered saline; PAR, protease-activated receptor.

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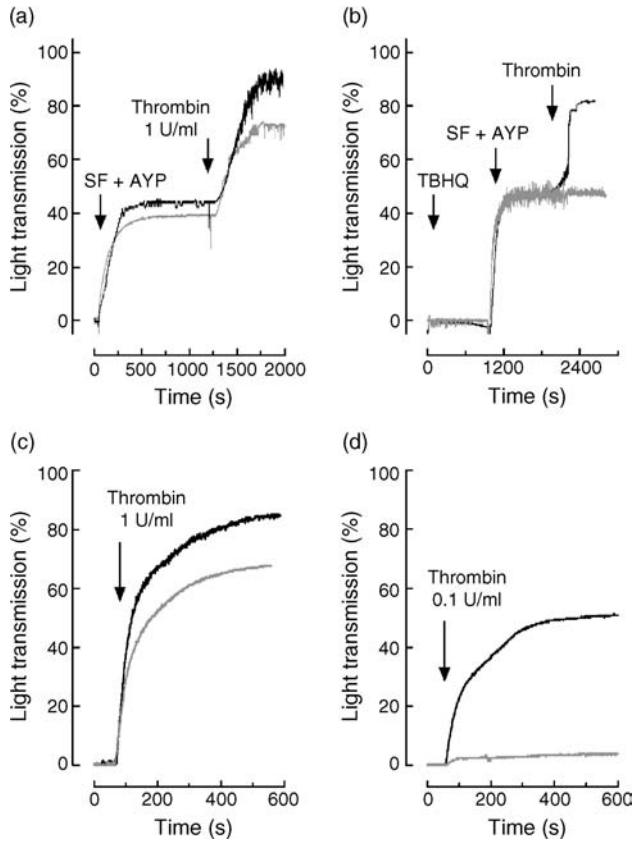
aggregation that was of greater amplitude than that induced by SFLLRN (Fig. 3a). AYPGKF-induced response was also found to be dependent on extracellular Ca^{2+}. As shown in Fig. 3b, acidic store depletion with TBHQ prior to the stimulation with 500 μmol/l AYPGKF reduced AYPGKF-induced platelet aggregation both in the presence and absence of extracellular Ca^{2+} (Fig. 3b, $P < 0.05$, $n = 6$).

The contribution of the leucine-rich glycoprotein receptor GPIb-IX-V in aggregation was investigated by desensitization of PAR-1 and PAR-4 receptors as previously described [24]. PAR-1 and PAR-4 receptors were desensitized by treatment with a combination of 10 μmol/l SFLLRN and 500 μmol/l AYPGKF as confirmed by the lack of effect of the PAR agonists SFLLRN and AYPGKF to induce platelet aggregation under these circumstances (data not shown). Stimulation of PAR-1 and PAR-4-desensitized platelets with thrombin 1 U/ml, to activate the GPIb-IX-V receptor, stimulated aggregation (Fig. 4a and Table 1). Platelet aggregation induced by sequential activation of the thrombin receptors reached amplitude that was found to be closer to that observed in platelets stimulated with thrombin itself (Fig. 4a). The response was significantly reduced when the cells were suspended in a Ca^{2+}-free medium (Fig. 4a; $P < 0.05$, $n = 6$). We note that the response induced by AYPGKF was similar to that observed with a combination of SFLLRN and AYPGKF in medium containing 1 mmol/l Ca^{2+}, suggesting that AYPGKF and SFLLRN might share a common signalling pathway. In cells pretreated with TBHQ to deplete the acidic stores the response to SFLLRN in combination with AYPGKF was similar in the presence or absence of extracellular Ca^{2+} (Fig. 4b), in contrast to the effects observed in platelets not treated with TBHQ (Fig. 4a). Pretreatment with TBHQ reduced GPIb-IX-V receptor-induced platelet aggregation in the presence of 1 mmol/l external Ca^{2+}, and abolished GPIb-IX-V receptor-induced response in the absence of extracellular Ca^{2+}, which is consistent with our previous studies reporting that activation of GPIb-IX-V by thrombin releases Ca^{2+} mostly from the acidic compartments in human platelets [23]. To confirm the role of the GPIb-IX-V receptor in platelet aggregation we used the anti-GPIb monoclonal antibody AK2, which has been shown to prevent thrombin binding to GPIb-IX-V [15]. Platelets were incubated with 5 μg/ml anti-GPIb monoclonal antibody AK2 or an identical concentration of unrelated mouse immunoglobulin for 2 min and then stimulated with either 1 or 0.1 U/ml thrombin. As shown in Fig. 4c and d, incubation with the anti-GPIb monoclonal antibody AK2 reduced platelet aggregation stimulated by both 1 and 0.1 U/ml thrombin, although this treatment was more effective at low thrombin concentrations.

Fig. 4



Platelet aggregation by activation of GPIb-IX-V receptor. (a and b) Human platelets were suspended in HBS containing 1 mmol/l Ca^{2+} (black traces) or in a Ca^{2+}-free HBS (grey traces) and preincubated for 8 min in the absence (a) or presence (b) of 20 μmol/l TBHQ. Cells were then stimulated with 10 μmol/l SFLLRN (SF) in combination with 500 μmol/l AYPGKF (AYP) followed by treatment with 1 U/ml thrombin. (c and d) Human platelets were suspended in Ca^{2+}-free HBS and preincubated for 2 min in the presence of 5 μg/ml anti-GPIb monoclonal antibody AK2 (grey traces) or identical concentration of unrelated mouse IgG, as control (black traces). Cells were then stimulated with 1 U/ml (c) or 0.1 U/ml (d) thrombin. Aggregation of human platelets was induced at a shear rate of 1200 r.p.m. at 37°C in an aggregometer as described in Materials and methods section. Traces shown are representative of six separate experiments. HBS, Hepes-buffered saline.

Discussion

Platelets increase $[Ca^{2+}]_c$ by releasing Ca^{2+} from intracellular compartments and facilitating the entry of Ca^{2+} through plasma membrane channels, a process that is required for full platelet activation including shape change, granular secretion, cytoskeleton remodelling and aggregation [24]. Platelets can be activated by a number of physiological agonists such as thrombin, which acts through a family of G-coupled PAR, PAR-1 and PAR-4 and the leucine-rich glycoprotein receptor GPIb-IX-V [15–17]. Since the description of two Ca^{2+} stores in platelets releasable by different agonists [25], recent work has showed that the TBHQ-sensitive Ca^{2+} store in human platelets is mainly a lysosomal-like acidic compartment [13]. We have recently reported that the DTS is required for platelet aggregation, although selective depletion of

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the DTS requires Ca^{2+} entry to activate full platelet aggregation [20].

Here we investigate the implication of acidic store in thrombin-induced aggregation. Treatment with TBHQ induces a shape change but not platelet aggregation by itself (data not shown). We have previously reported that TBHQ enhances a small elevation in $[Ca^{2+}]_c$ due to release of Ca^{2+} from the acidic stores, followed by store-operated Ca^{2+} entry, that was smaller in magnitude than that induced by discharge of the DTS [26]. The lack of effect of TBHQ on platelet aggregation by itself might be due to the small increase in $[Ca^{2+}]_c$ resulting from its effect on platelets. As previously reported [20], a significant elevation in $[Ca^{2+}]_c$ is necessary for platelet aggregation. In addition, the lack of effect of TBHQ inducing aggregation *per se* might also be explained by inhibition of protein tyrosine phosphorylation [27], cytoskeleton remodelling [28–30] and TxB2 formation [31]. Now, we show that Ca^{2+} accumulated into the acidic stores is important for platelet aggregation induced by thrombin or sequential activation of the thrombin receptors. We performed experiments with SFLLRN and AYPGKF, two selective and strong agonist peptides of PAR-1 and PAR-4, respectively. Our results indicate that the two agonists enhance only a small aggregation in agreement with previous studies [20,23]. SFLLRN and AYPGKF-induced aggregation was reduced in the absence of calcium. Interestingly pretreatment of platelets with 20 μmol/l THBQ reduced the difference between SFLLRN-stimulated platelets in the presence or absence of extracellular Ca^{2+} but not in AYPGKF-stimulated platelets. This result can be explained by a decrease in calcium re-uptake and the maintenance of a relatively high $[Ca^{2+}]_c$ in SFLLRN-stimulated platelets that overcomes the absence of calcium influx. But in AYPGKF-stimulated platelets, the integrity of acidic stores seems to be necessary for AYPGKF-mediated response.

In a Ca^{2+}-free medium, treatment with SFLLRN and AYPGKF induces a transient increase of intracellular calcium which return to the basal level through the endomembrane Ca^{2+}-ATPase pumps and resulted in a smaller response than in the presence of extracellular Ca^{2+}. Those findings suggest that calcium entry might be required for aggregation, by amplification of the internal signal generated by store depletion. In accordance with this, we have previously demonstrated that aggregation needs a high calcium concentration perhaps to activate a low-affinity Ca^{2+}-dependent pathway [20]. So the intracellular $[Ca^{2+}]_c$ achieved by TBHQ was only sufficient to cause shape change, but not enough to give aggregation.

Stimulation of platelets in a medium containing 1 mmol/l Ca^{2+} with SFLLRN in combination with AYPGKF induces a response that was similar to that observed with AYPGKF alone suggesting that aggregation-induced by activation of PAR-1 and PAR-4 might share a common signal transduction pathway.

Platelet aggregation induced by activation of the GPIb-IX-V receptor was abolished by previous depletion of the acidic stores when cells were stimulated in a Ca^{2+}-free medium. These findings are consistent with our previous studies reporting that activation of the GPIb-IX-V receptor by thrombin releases Ca^{2+} solely from the acidic compartments [23]. In the presence of extracellular Ca^{2+} pretreatment with TBHQ reduced platelet aggregation induced by activation of GPIb-IX-V by only 20%. The different effects observed in the absence or presence of extracellular Ca^{2+} might suggest the involvement of a Ca^{2+}-dependent intracellular pathway in this process. Despite the release of Ca^{2+} accumulated in the acidic compartments is unable to induce platelet aggregation *per se*, our results indicate that it is important for thrombin-induced aggregation through the three receptor types described in human platelets.

The role of GPIb-IX-V in thrombin-evoked platelet aggregation was confirmed by incubation with the anti-GPIb monoclonal antibody AK2, which reduced platelet aggregation stimulated by both 1 and 0.1 U/ml thrombin. The fact that this treatment was more effective at low thrombin concentrations further confirms that the GPIb-IX-V receptor shows high affinity for thrombin [32,33]. In addition, these findings confirm previous studies reporting that Ca^{2+} mobilization mediated by activation of GPIb-IX-V by thrombin is entirely dependent on Ca^{2+} accumulated in the acidic stores, which are completely discharged at low thrombin concentrations that induce Ca^{2+} release almost entirely from the acidic stores [23]. The inhibition of platelet aggregation with 1 U/ml thrombin induced by the anti-GPIb monoclonal antibody AK2 was similar to the extent of aggregation that remained after application of the PAR-1 and PAR-2 agonists, abolished by TBHQ (see Fig. 4c vs. Fig. 4b), further suggesting a role for the acidic stores and the GPIb-IX-V in thrombin-induced platelet aggregation.

In conclusion we report that Ca^{2+} entry seems to be necessary for platelet aggregation upon activation of thrombin receptors. In addition, Ca^{2+} accumulation in the acidic stores is required for platelet aggregation induced by activation of the G-coupled PAR-1 and PAR-4 receptors and, especially by the occupation of the leucine-rich glycoprotein GPIb-IX-V, whose Ca^{2+} mobilization is entirely dependent on the acidic stores [23]. These findings provide evidence for a functional role of the lysosomal-like acidic Ca^{2+} stores in human platelets.

Acknowledgements

The work was supported by Tunisian-Spanish PCI project (A/8203/07). We thank Mercedes Gómez Blázquez for her technical assistance.

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