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Thrombin-sensitive expression of the store operated Ca^{2+} channel Orai1 in platelets

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ABSTRACT

Thrombin activates pore forming channel protein Orai1 resulting in store operated Ca^{2+} entry (SOCE) with subsequent Ca^{2+}-dependent release of platelet granules, activation of integrin $\alpha_{IIb}\beta_3$, adhesion, aggregation and thrombus formation. Platelets lack nuclei and are thus unable to modify protein abundance by transcriptional regulation. Nevertheless, they still contain pre-mRNA and mRNA and are thus able to express protein by stimulation of rapid translation. Platelet translation is sensitive to phosphoinositide-3-kinase (PI3K) and actin polymerization. The present study explored whether platelet activation via thrombin modifies Orai1 protein abundance. According to RT-PCR platelets contain pre-mRNA and mRNA encoding Orai1. Activation with thrombin (0.1 U/ml) results in a significant decline of pre-mRNA, which is, according to Western blotting and confocal microscopy, paralleled by a marked and statistically significant increase of Orai1 protein abundance. The increase of Orai1 protein abundance is insensitive to inhibition of transcription with actinomycin (4 μg/ml), but is significantly blunted by inhibition of translation with puromycin (100 nM) and by inhibition of PI3K with wortmannin (100 nM) or LY294002 (25 μM). In conclusion, activation of platelets stimulates the translational expression of Orai1, thus augmenting platelet Ca^{2+} signaling.

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1. Introduction

Platelets are decisive for primary hemostasis following vascular injury but also critical for the development of acute thrombotic occlusion at regions of atherosclerotic plaque rupture [1,2] leading to ischemic diseases, such as myocardial infarction or ischemic stroke [3]. Moreover, platelets play a decisive role in the pathogenesis of several inflammatory diseases, particularly in vascular inflammation and atherogenesis [2,4].

Activation of platelets with thrombin is followed by stimulation of exocytosis, activation of integrin $\alpha_{IIb}\beta_3$ and triggering of adhesion, aggregation and thrombus formation [5]. All these functions are critically dependent on an increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) [6,7]. Platelet activation by collagen or thrombin leads to an activation of phospholipase $C_\gamma 2$ ($PLC_\gamma 2$) or phospholipase C_β (PLC_β). As a consequence phosphatidylinositol-4,5-bisphosphate (PIP_2) dissociates into inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [5]. Whereas DAG is responsible for the activation of TRPC-channels [8], IP_3 activates the ryanodine-receptors in the endoplasmatic reticulum (ER) and thus leads to a Ca^{2+}-release into the cytosol [5]. Due to this activation dependent Ca^{2+} release from intracellular stores the activated platelets undergo a cytoskeletal reorganization which by itself is responsible for the shape change [9]. Simultaneously the stromal interaction molecule 1 (STIM1) is activated by emptying of the intracellular Ca^{2+} stores [10]. STIM1 is localized in the ER and contains a Ca^{2+} binding domain (EF-hand) in the ER-lumen and is activated by Ca^{2+}-depletion of the intracellular stores [10]. In its activated form STIM1 undergoes a conformational change via sterical forces and can thus interact with Orai1 in the plasma membrane upon store depletion [11]. Four of those Orai1-molecules combine to the pore forming Ca^{2+} release-activated channel (CRAC) Orai1 (CRACM1). Depletion of intracellular stores and the subsequent stimulation of Orai1 thus causes the so-called store operated calcium entry (SOCE) [12,13]. STIM1-mediated Orai1-activation leads to a sustained influx of Ca^{2+} into the activated cell and plays the major role in platelet activation [5,6,14].

Although Orai1 plays a crucial role in platelet physiology, the regulation of Orai1 is still incompletely understood. Regulators of Orai1 protein abundance and thus platelet SOCE include the serum- and glucocorticoid-inducible kinase isoforms SGK1 [1,15] and SGK3 [16]. SGK1 stimulates Orai1 transcription in megakaryocytes by upregulating Orai1 transcription factor NF-κB [1]. Whether or not Orai1 is translated in mature platelets, remained elusive.

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Circulating platelets are devoid of nuclei and thus cannot transcribe novel proteins [17]. However, platelets harbour pre-mRNA and mRNA due to the megakaryopoetic process and after activation platelets are able to splice the intronic rich pre-mRNA into mature mRNA with following translation into mature proteins [18–20]. In recent years it could be conclusively shown that after coactivation with fibrinogen and thrombin platelets do synthesize *de novo* a few important platelet proteins such as interleukin-1β (IL-1β) [21] and human tissue factor (TF) [22]. Important regulators of translation include phosphatidylinositide-3-kinase (PI3K) [20] and cytoskeletal reorganization [23].

Prior to stimulation of translation, the mRNA associates with the cytoskeletal core and the eukaryotic initiating factor eIF-4E localizes to the membrane skeleton and the soluble fraction of platelets [21]. Moreover, eIF-4E is bound to the inhibitory 4E-BP1 molecule and is thus prevented to initiate translation [24]. Activation of platelets is followed by association of PI3K with the membrane skeleton [23] and subsequent phosphatidylinositide-3-kinase (PI3K) dependent phosphorylation of 4E-BP1 [20,24]. Based on this phosphorylation the inhibitory binding molecules dissociates and the translation initiation factors can redistribute to the proximity of mRNA [21] with resulting translation of mRNA.

The present study explored whether activation of platelets by thrombin modifies the expression and regulation of the Orai1 protein. Accordingly, pre-mRNA and mRNA levels were quantified by RT-PCR and protein abundance was quantified by Western blotting and confocal microscopy in platelets prior to and following activation with thrombin. Further experiments were performed in the presence or absence of transcription inhibitor actinomycin, translation inhibitor puromycin as well as PI3K inhibitors Wortmannin and LY294002.

2. Materials and methods

2.1. Preparation of human platelets

Human platelets were isolated as described previously [25]. Blood from healthy volunteers was collected in ACD-buffer and centrifuged at 200*g* for 20 min. The obtained platelet-rich plasma was added to modified Tyrode-HEPES (N-2-hydroxyethyl-piperazone-Ń2-ethanesulfonic acid) buffer (137 mM NaCl, 2.8 mM KCL, 12 mM NaHCO3, 5 mM glucose, 0.4 mM Na_2HPO_4, 10 mM HEPES, 0.1% bovine serum albumin, pH 6.5). After centrifugation at 900*g* for 10 min and removal of the supernatant, the resulting platelet pellet was resuspended in Tyrode–HEPES buffer (pH 7.4, supplemented with 1 mM $CaCl_2$). Afterwards platelets were activated using thrombin (0.1 U/ml; Roche, Basel, Switzerland).

2.2. RT-PCR analysis

To determine Orai1 mRNA and pre-mRNA abundance in platelets RNA was extracted from 10^8 cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Subsequently approximately 2 μg of total RNA was reverse transcribed to cDNA using random hexamer primers (0.1 mM, Roche), 1st strand buffer (Invitrogen), DTT (10 mM, Invitrogen) and SuperScript II Reverse Transcriptase (200 U, Invitrogen) for 1 h at 42 °C. Quantitative real-time PCR was applied on the CFX96 Real-Time System C1000 Thermal Cycler (Biorad) using the following primer pairs (5′–3′ orientation): Orai1 mRNA forward TGA TGA GCC TCA ACG AGC ACT CCA TG and Orai1 mRNA reverse TGC TGA TCA TGA GCG CAA ACA GGT G.

For the detection of Orai1 pre-mRNA a nested PCR was performed [26]. In a first step a larger cDNA fragment was amplified with the following primers (5′–3′ orientation): forward GCT AGG ACT GAA GAG TAG TAG TGT and reverse CTC CTT GAC CGA GTT GAG ATT G. Afterwards a second PCR was performed with the product of the first PCR added instead of the cDNA using as a forward primer GCA CTC ATC CTG CCT GTC and as a reverse primer GTA GTC GTG GTC AGC GTC.

The transcript levels of the house-keeping gene *Gapdh* were determined for each sample using the following primers (5′–3′ orientation): forward TGA GTA CGT CGT GGA GTC CAC TG and reverse TTC TGG GTG GCA GTG ATG GCA TG. Amplification of the house-keeping gene *Gapdh* was performed to standardize the amount of RNA per sample.

2.3. Western blot analysis

Human platelets were isolated as described above and pellets of 10^8 cells were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Trion-X, 0.5% Na_2HPO_4, 0.4% β-Mercaptoethanol) containing protease inhibitor cocktail (Sigma–Aldrich). After centrifugation for 15 min at 20,000*g* and 4 °C the pellets were trashed and the supernatant was taken for Western blot analysis.

The protein concentration was measured with Bradford protein assay (Biorad) and finally 50 μg of protein were taken for each sample. Lysis buffer was added to get a uniform sample concentration and the samples were boiled for 5 min at 95 °C. For immunoblotting proteins were electrotransferred onto a PVDF membrane and blocked with 5% bovine serum albumin in TBS with 0.1% Tween 20 (TBST) at room temperature for 1 h. Afterwards the membrane was incubated with the primary antibody against β-Actin (1:1000, Cell Signaling) or Orai1 (1 μg/ml, Millipore) at 4 °C overnight. These data were confirmed by using a second antibody against Orai1 (Abcam). After washing the membrane with TBST the membranes were incubated with secondary antibody conjugated with horse radish peroxidase (HRP) (1:1000, Cell Signaling) for 1 h at room temperature. After washing antibody binding was detected with the ECL detection reagent (Amersham). Blots were then exposed to photographic films and the optical density was estimated using scanning densitometry.

2.4. Immunofluorescence and confocal microscopy

Isolated human platelets were attached to a fibrinogen coated surface (20 μg/ml) on a chamber slide. Adherent platelets were fixed with 2% paraformaldehyde, washed and blocked with BSA (2%) for at least 30 min. Additionally, the platelets were permeabilized using 0.15% Triton-X treatment for 10 min. Finally, the primary antibody against Orai1 (1:1000 Millipore) was incubated for 2 h at room temperature. After washing of the chamber slides they were incubated with a FITC-conjugated secondary antibody (1:500, Santa Cruz Biotechnology) and the actin cytoskeleton was stained with rhodamine-phalloidin (Invitrogen). The slides were mounted with ProLong Gold antifade reagent (Invitrogen). The platelets were analysed by confocal microscopy using a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss Micro Imaging) with an A-Plan 63x ocular.

2.5. Statistical analysis

Data are provided as means ± SEM; *n* represents the number of independent experiments. All data were tested for significance using ANOVA Dunnett's test. Only results with $^{*}(p < 0.05)$ and $^{**}(p < 0.01)$ respectively $^{\#}(p < 0.05)$ and $^{\#\#}(p < 0.01)$ were considered statistically significant.

3. Results

According to Western blot analysis human platelets strongly express Orai1 protein and activation of platelets with thrombin (0.1 U/ml) for 15, 30 and 60 min resulted in a marked, statistically significant increase of Orai1 protein abundance (Fig. 1 A and B). These results were confirmed by confocal microscopy. As shown in Fig. 1 C platelet activation with 0.1 U/ml thrombin for 15, 30 and 60 min were followed by an increase of the Orai1 protein abundance.

Since PI3K has been shown to regulate protein expression levels in activated platelets, additional experiments explored whether the increase of Orai1 protein abundance following thrombin stimulation was sensitive to phosphoinositide-3-kinase (PI3K). To this end, human platelets were stimulated with 0.1 U/ml thrombin in the absence and presence of the PI3K inhibitors Wortmannin (Wm; 100 nM) or LY294002 (25 μM). As shown in Fig. 2 the significant increase in Orai1 protein abundance after thrombin stimulation was indeed significantly blunted by preincubation of the platelets with the PI3K inhibitors Wortmannin and LY294002 (Fig. 2).

To examine the influence of the transcriptional or translational process on activation-dependent upregulation of Orai1 expression in anucleated platelets the samples were treated prior to thrombin stimulation with the transcription inhibitor actinomycin or with the translation inhibitor puromycin. As shown by Western blot analysis disruption of transcription with actinomycin (4 μg/ml) did not significantly modify the increase of activation dependent Orai1 protein abundance whereas puromycin (100 μM), a highly potent inhibitor of cellular translation processes, significantly blunted the activation dependent increase of Orai1 protein abundance upon thrombin stimulation (Fig. 3). These observations point to a critical role of translation in the upregulation of platelet Orai1 expression upon thrombin-triggered platelet stimulation.

In a further series of experiments the Orai1 pre-mRNA and RNA levels were determined in human platelets prior to and after thrombin stimulation. According to reverse transcription polymerase chain reaction (RT-PCR) pre-mRNA and mRNA encoding the pore forming Ca^{2+} release-activated channel (CRAC) Orai1 were expressed in circulating human platelets (Fig. 4). As shown in Fig. 4, activation of platelets with thrombin (0.1 U/ml) for different time points resulted in a rapid decline of pre-mRNA levels and a statistically significant increase of mRNA levels pointing to a splicing from the Orai1 intronic rich pre-mRNA into mature Orai1 mRNA (Fig. 4). The increase of Orai1 protein quantity after thrombin stimulation contrasted the decline of transcript levels and was therefore not caused by enhanced Orai1 transcription.

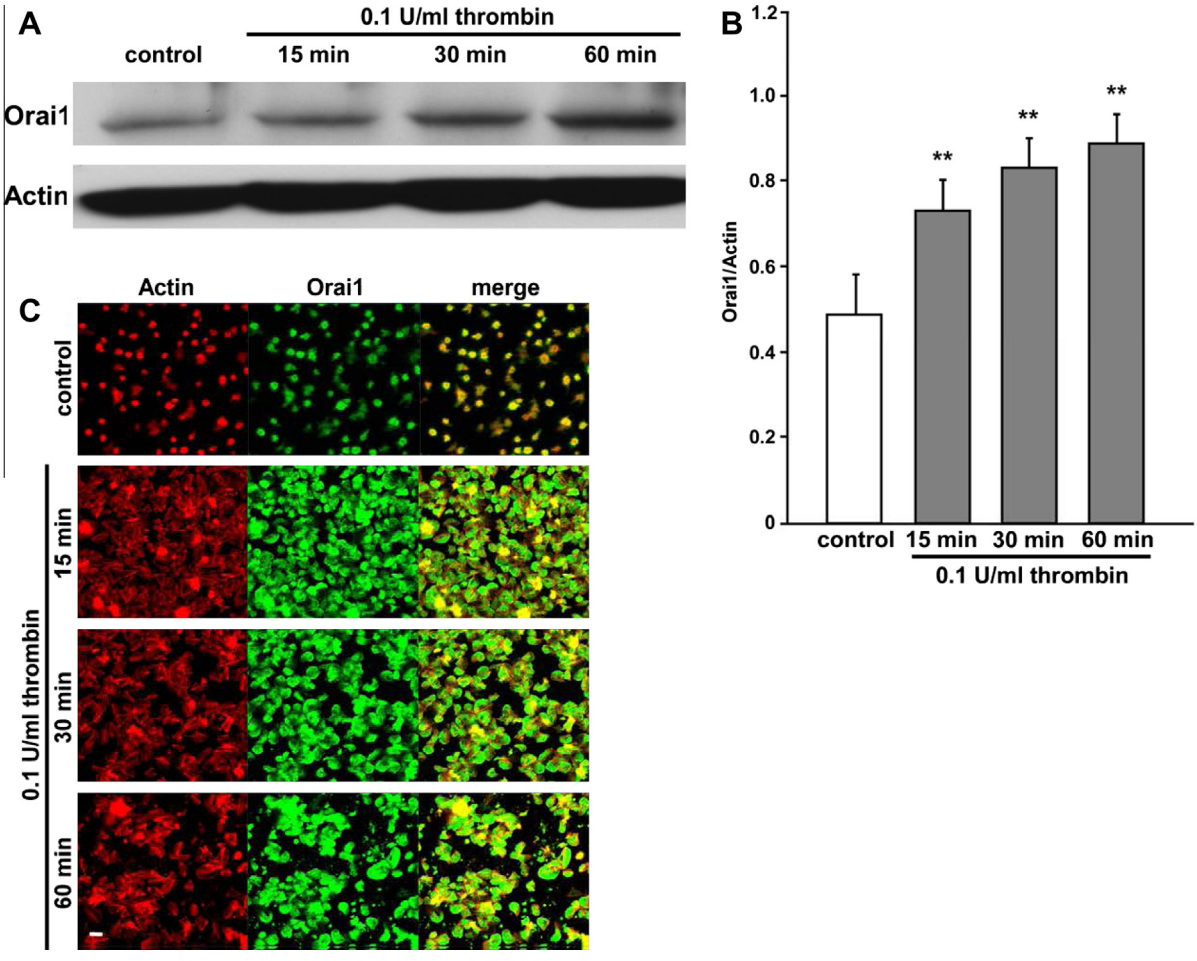

Fig. 1. Activation-dependent Orai1 protein expression in platelets upon thrombin-triggered stimulation. (A) Original Western blot of Orai1 protein abundance in human platelets prior to and following administration of thrombin (0.1 U/ml) for the indicated time periods. (B) Arithmetic means ± SEM ($n = 5$) of Orai1 protein abundance in human platelets prior to (white bar) and following (grey bars) administration of thrombin (0.1 U/ml) for the indicated time periods. **($p < 0.01$) indicates statistically significant differences compared with the value prior to thrombin administration. (C) Original confocal microscopy of Orai1 abundance in human platelets prior to and following activation of thrombin (0.1 U/ml) for the indicated time periods. Scale bar represent 5 μm. Green: Orai1, red: actin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

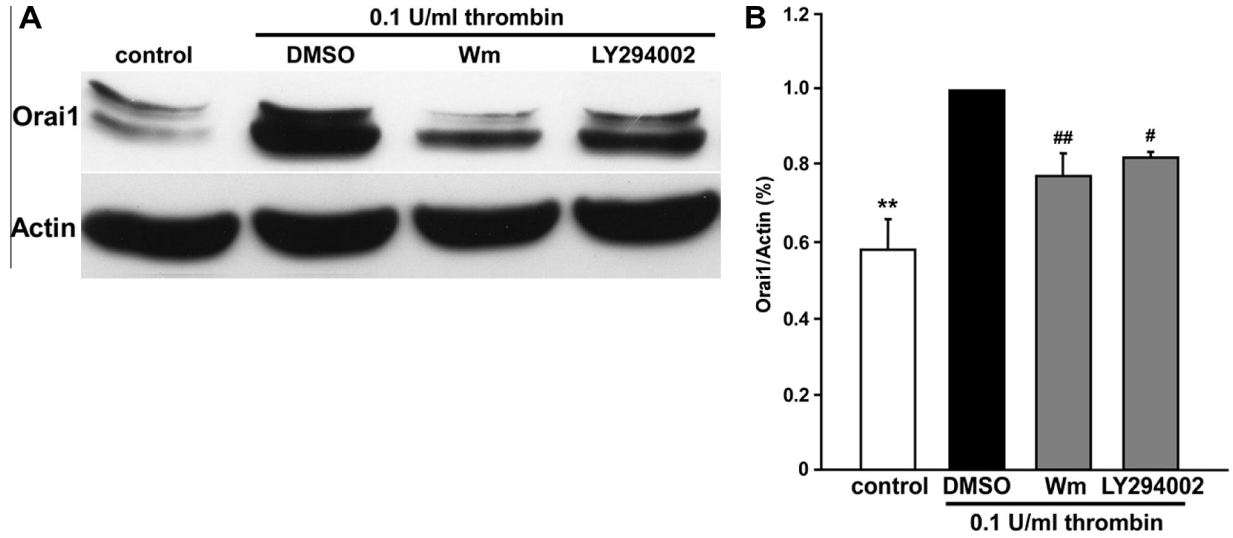

Fig. 2. Effect of PI3K inhibitors on activation-dependent upregulation of Orai1 protein expression in platelets. (A) Original Western blot of Orai1 protein abundance in human platelets prior to and following administration of thrombin (0.1 U/ml) in the absence or presence of Wortmannin (100 nM) or LY294002 (25 μM). (B) Arithmetic means ± SEM ($n = 5$) of Orai1 protein abundance in human platelets prior to (white bar) and following (black and grey bars) administration of thrombin (0.1 U/ml) in the absence (black bar) and presence of Wortmannin (100 nM, grey bar) or LY294002 (25 μM, grey bar). $^{**}(p < 0.01)$ indicates statistically significant difference compared with the value prior to thrombin administration, $^{\#}(p < 0.05)$ and $^{\#\#}(p < 0.01)$ indicates statistically significant differences compared with the value in the absence of inhibitor.

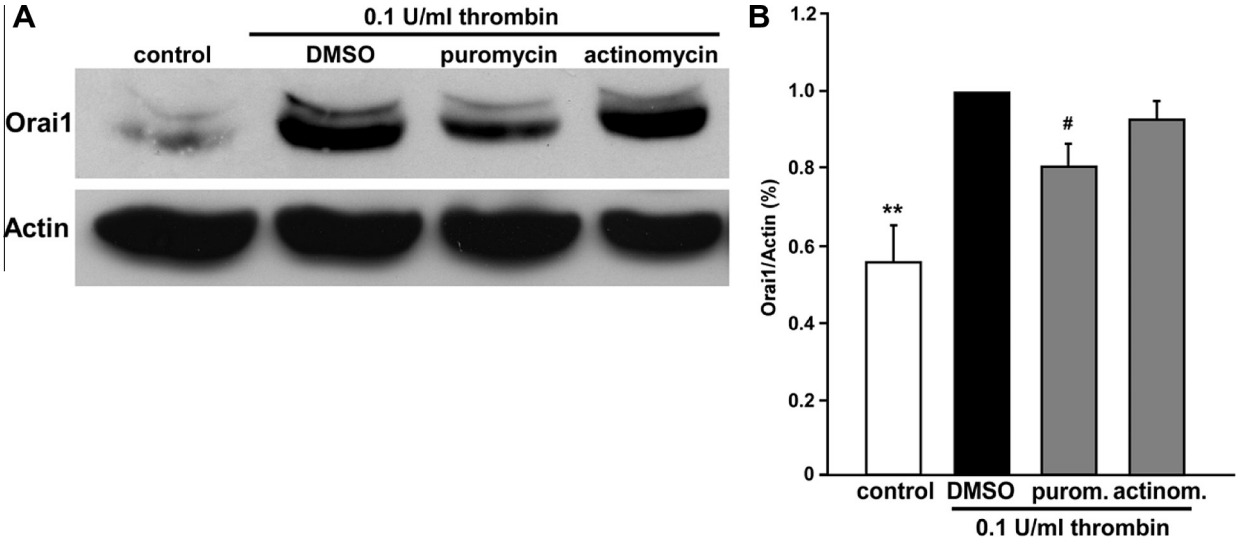

Fig. 3. Effect of transcription and translation inhibitors on activation-dependent upregulation of Orai1 protein expression in platelets. (A) Original Western blot of Orai1 protein abundance in human platelets prior to and following administration of thrombin (0.1 U/ml) in the absence or presence of actinomycin (4 μg/ml) or puromycin (100 nM) for the indicated time periods. (B) Arithmetic means ± SEM ($n = 6$) of Orai1 protein abundance in human platelets prior to (white bar) and following (black and grey bars) administration of thrombin (0.1 U/ml) in the absence (black bar) and presence of actinomycin (4 μg/ml, grey bar) or puromycin (100 nM, grey bar). $^{**}(p < 0.01)$ indicates statistically significant difference compared with the value prior to thrombin administration, $^{\#}(p < 0.05)$ indicates statistically significant difference compared with the value in the absence of inhibitor.

4. Discussion

According to the present study platelets are able to translationally upregulate the pore forming Ca^{2+} release-activated channel Orai1 following activation by thrombin. The translation of Orai1 protein is sensitive to actin polymerization and to activation of phosphoinositide-3-kinase (PI3K). According to earlier studies PI3K activation is of pivotal importance for platelet activation [27].

The degranulation, integrin $\alpha_{IIb}\beta_3$ activation and adhesion of platelets with the subsequent thrombus formation are triggered by an increase in $[Ca^{2+}]_i$ [6], which results from the release of Ca^{2+} from intracellular stores and following activation of store operated Ca^{2+} entry (SOCE) by store depletion [5]. SOCE results from stimulation of the pore forming Ca^{2+} release activated Ca^{2+} channel (CRAC) Orai1 [28,29] by STIM1 [30], a sensor of the Ca^{2+} content of the sarcoplasmatic reticulum [31]. Platelet activation critically depends on functional Orai1 and STIM1 [14,31,32].

Orai1 translation presumably involves the translation initiation factors elF-4E and elF-2alpha eIF-4E [21,33] as well as the inhibitory 4E binding protein 4E-BP1 [21]. Prior to stimulation of translation, the mRNA associates with the cytoskeletal core and eIF-4E localizes to the membrane skeleton and the soluble fraction of platelets [21]. Moreover, eIF-4E is bound to 4E-BP1 and thus prevented to initiate translation [24]. Activation of the platelets is followed by redistribution of the translation initiation factors to the proximity of mRNA [21] and by association of PI3K with the

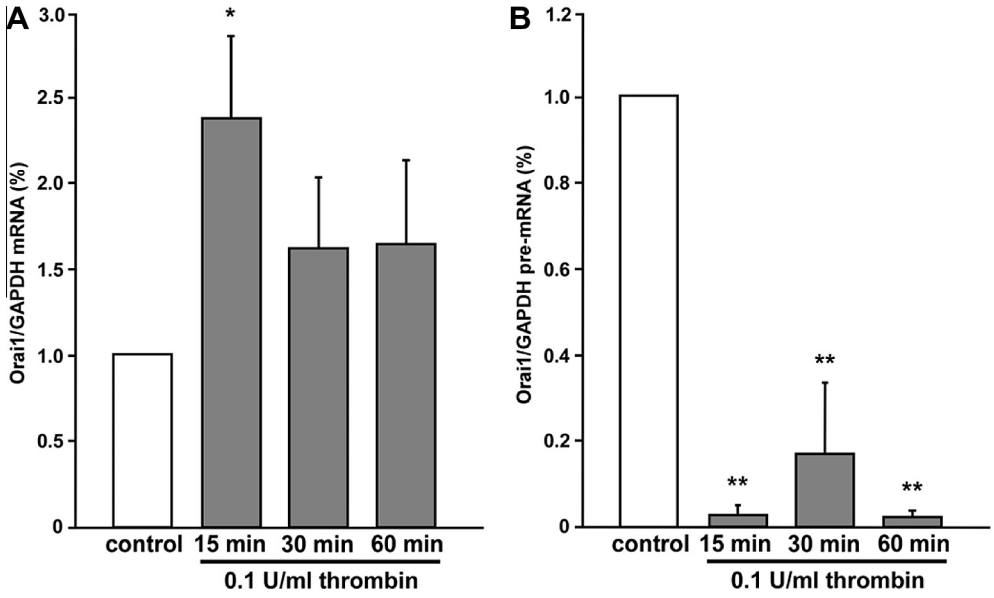

Fig. 4. Activation-dependent Orai1 pre-mRNA and mRNA abundance in platelets upon thrombin-triggered platelet stimulation. (A) Arithmetic means ± SEM ($n = 4$) of Orai1 mRNA abundance in human platelets prior to (white bar) and following (grey bars) administration of thrombin (0.1 U/ml) for the indicated time periods. *($p < 0.05$) indicates statistically significant difference compared with the value prior to thrombin administration. (B) Arithmetic means ± SEM ($n = 5$) of Orai1 pre-mRNA abundance in human platelets prior to (white bar) and following (grey bars) administration of thrombin (0.1 U/ml) for the indicated time periods. **($p < 0.01$) indicates statistically significant difference compared with the value prior to thrombin administration.

membrane skeleton [23] resulting in PI3K dependent phosphorylation of 4E-BP1 [24]. The present observations revealed that both actin polymerization and PI3K activation are required for the triggering of Orai1 translation. The upregulation of Orai1 is expected to boost the capacity of platelets to accomplish hemostasis [34], as Orai1 deficient mice suffer from severe bleeding [14].

In conclusion, activation of platelets by thrombin leads to upregulation of Orai1 protein translation which is expected to enhance activation-dependent Ca^{2+} entry into platelets with subsequent Ca^{2+}-dependent degranulation, adhesion, aggregation and thrombus formation.

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