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Mouse transient receptor potential channel type 6 selectively regulates agonist-induced platelet function

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ABSTRACT

While changes in intracellular calcium levels is a central step in platelet activation and thrombus formation, the contribution and mechanism of receptor-operated calcium entry (ROCE) via transient receptor potential channels (TRPCs) in platelets remains poorly defined. In previous studies, we have shown that TRPC6 regulates hemostasis and thrombosis, in mice. In the present studies, we employed a knockout mouse model system to characterize the role of TRPC6 in ROCE and platelet activation. It was observed that the TRPC6 deletion (*Trpc6*$^{-/-}$) platelets displayed impaired elevation of intracellular calcium, i.e., defective ROCE. Moreover, these platelets also exhibited defects in a host of functional responses, namely aggregation, granule secretion, and integrin αIIbβ3. Interestingly, the aforementioned defects were specific to the thromboxane receptor (TPR), as no impaired responses were observed in response to ADP or the thrombin receptor-activating peptide 4 (TRAP4). The defect in ROCE in the Trpc6$^{-/-}$ was also observed with 1-oleoyl-2-acetyl-sn-glycerol (OAG). Finally, our studies also revealed that TRPC6 regulates clot retraction. Taken together, our findings demonstrate that TRPC6 directly regulates TPR-dependent ROCE and platelet function. Thus, TRPC6 may serve as a novel target for the therapeutic management of thrombotic diseases.

1. Introduction

Platelet activation plays an important role in physiologic hemostasis, whereas hyperactive platelets are known to lead to thrombotic events such as myocardial infarction or stroke. Platelet activation is initially triggered by subendothelial matrix such as collagen, and subsequently by secondary mediators such as thromboxane A_2 (TXA_2), and adenosine diphosphate (ADP), released by activated platelets [1,2]. Although these agonists stimulate different signaling pathways, all of them induce the activation of phospholipase C (PLC) isoforms resulting in the hydrolysis of phospatidlylinositol 1–4, 5-biphosphate (PIP2) to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) [3]. This results in the release of Ca^{2+} from intracellular stores and subsequent Ca^{2+} influx through plasma membrane Ca^{2+} channels, leading to an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), and consequent platelet activation. There are three types of Ca^{2+} channels in the platelet plasma membrane: (i) store-operated calcium (SOC) channels regulated by the filling state of the intracellular stores, (ii) non-SOC channels activated by multiple modalities and (iii) receptor-operated calcium (ROC) channels opening through ligand binding [4]. Receptor activation results in Ca^{2+} release from the intracellular stores, leading to an opening of the plasmatic Ca^{2+} channels and extracellular Ca^{2+} entry into the cell [5]. Moreover, Ca^{2+} signaling dysfunction may lead to the pathogenesis of several platelet-linked disorders [6]. To this end, abnormal Ca^{2+} signals in response to physiological agonists have been associated with platelet malfunction, suggesting a role for Ca^{2+} in the pathogenesis of thromboembolism [2,7,8].

The canonical transient receptor potential channel (TRPC) family of proteins has garnered a lot of attention as a mediator of ROC entry (ROCE), with TRPC6 shown to be activated by DAG in different cell types [9], including human platelets [10–12]. In fact, TRPC6 have been identified as the channels that through 1-oleoyl-2-acetyl-sn-glycerol (OAG)–induced external calcium entry, accelerate the activation and inactivation of human platelets [13–15]. We have previously shown that TRPC6 deficient mice exhibit increased bleeding and occlusion time than their wild type (WT) littermates [16]. Moreover, we also

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found that a TRPC6 inhibitor selectively inhibited TXA_2 receptor (TPR)-induced platelet function. Nonetheless, whether TRPC6 plays a direct role in regulating platelet function remains to be confirmed. In the present study, we sought to further investigate the role of TRPC6 in platelet ROCE, and the importance of such regulation in platelet function, by employing the *Trpc6*$^{-/-}$ mice. Our results indicate that the absence of TRPC6 induces a TPR/agonist-specific decrease in ROCE, in platelet. This decrease in ROCE is associated, again, in a TPR/agonist-selective manner, with impaired platelet aggregation, secretion, integrin αIIβ3 activation, and clot retraction.

Collectively, our studies support the notion that TRPC6 regulates TPR-mediated ROCE, and plays an important role in platelet function. Consequently, TRPC6 may be an attractive target for managing thrombotic diseases.

2. Materials and methods

2.1. Reagents and materials

Antibodies against TRPC6 and control peptides were from Alomone Labs (Jerusalem, Israel). Anti-β Actin was form Millipore (Temecula, CA). RIPA Lysis Buffer and goat anti-rabbit IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphatase inhibitor cocktails 2 and 3, ionic detergent tween 20, indomethacin, EGTA, ADP, calcium chloride and 1-oleoyl-2-acetyl-sn-glycerol (OAG) were from Sigma Aldrich (St Louis, MO). BCA protein assay kit and ECL western blotting substrate were from Pierce Biotechnology (Rockford, IL). Molecular weight markers, ethidium bromide and immuno-blot PDVF membrane for protein blotting were from Bio-Rad (Hercules, CA). 6 X SDS Reducing Sample Buffer were from Boston Bioproducts (Ashland, MA). L-dithiotheriol (DTT) was form Promega (Madison, WI). U46619, thapsigargin and prostaglandin I_2 were from Cayman Chemical (Ann Arbor, MI). Prostaglandin E_1 (Alprostadil) was from Tocris (Minneapolis, MN). Thrombin, ATP and Luciferin-luciferase were from Chrono-Log (Havertown, PA). Selective Protease-Activated Receptor 4, PAR4 (AYPGKF-NH_2) activating peptide (TRAP4) was from Peptides International (Louisville, KY). Sodium citrate (3.8% w/v), Triton X-100 and D-dextrose were from Fisher Scientific (Hanover Park, IL). Hepes Buffered Saline (HBS) was from Fisher (Pittsburgh, PA). Bovine serum albumin (BSA) was from Equitech-Bio, Inc. (Kerrville, TX). Fura-2 acetoxymethyl ester (fura-2/AM) and Pluronic® F-127 were from Invitrogen (Grand Island, NY). Apyrase was from Biolabs (Ipswich, MA). FITC Hamster Anti-Mouse CD61, PE Rat Anti-Mouse CD41, FITC Rat Anti-Mouse CD62P, Mouse Fc Block (CD16-CD32), PE Rat Anti-Mouse IgG_{1k} and FITC Rat Anti-Mouse IgG_{1k} were from BD Pharmigen (San Diego, CA). Platelets were counted in an automatic hematology analyzer blood counter (Hemavet 958, Erba® Diagnostics (Miami Lakes, FL).).

2.2. Mice and genotyping

Trpc6$^{-/-}$ mice were from Dr. Lutz Birnbaumer (NIH) and were backcrossed for 10 generations onto a C57BL/6J background, and genotyped as described before [16,17], using a PCR-based method. PCR was performed using following primers: WT forward 5′-CAGATCATCT CTGAAGGTCTTTATGC-3′, and reverse 5′-TGTGAATGCTTCATTCTGTT TTGCGCC-3'. KO forward 5′-GGGTTTAATGTACTGTATCACTAAAGCC TCC and reverse 5′-ACGAGACTAGTGAGACGTGCTACTTCC-3′, and reverse with the following PCR condition: 94 °C (7 min) followed by 94 °C (1 min), 58 °C (1 min), and 72 °C (1 min 30 s) for 35 cycles. DNA were run in 1% agarose gel and visualized in the gel documentation system. The following products were observed: *Trpc6*$^{+/+}$: 254 bp, *Trpc6*$^{+/-}$: 254/310 bp, and *Trpc6*$^{-/-}$: 310 bp. Western blotting was performed in order to compare *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ platelets with regard to TRPC6 expression, in a 12% SDS-PAGE gel. Mice were housed in groups of 1–4 at 24 °C, under 12/12 light/dark cycles, with access to water and food ad libitum, with experiments performed at the age of 8–10 weeks (mixed gender), except the age-dependent expression analysis. All experiments involving animals were performed in compliance with the institutional guidelines, and were approved by the Institutional Animal Care and Use Committee.

2.3. Immunoblotting

Platelet proteins (50 μg), from whole cell lysates, from four, six and 10 week old mice, were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to Immobilon-P PVDF membranes, as previously described [18,19]. The blots were incubated with different antibodies: anti-TRPC6, anti-actin. Following washing, the blots were incubated with HRP-labeled anti-rabbit IgG or anti-mouse IgG as required. The antibody binding was detected using enhanced chemiluminescence substrate (Thermo Scientific, Rockford, IL). Images were obtained with ChemiDoc MP Imaging System (BioRad, Hercules, CA).

2.4. Platelet preparation

Mouse blood was collected from the ventricle and the citrated (0.38%) blood was mixed with phosphate-buffered saline, pH 7.4, and incubated with PGI_2 (10 ng/mL; 5 min), followed by centrifugation at 237 × *g* for 10 min at room temperature (RT). Platelet-rich plasma (PRP) was recovered and platelets were pelleted at 483 × *g* for 10 min at RT. The pellets were resuspended in HEPES/Tyrode's buffer (HT; 20 mM HEPES/KOH, pH 6.5, 128 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM D-glucose) supplemented with 1 mM EGTA, 0.37 U/mL apyrase, and 10 ng/mL PGI_2. Platelets were washed and resuspended in HT (pH 7.4) without EGTA, apyrase, or PGI_2. Platelets were counted with an automated hematology analyzer (Drew Scientific Dallas, TX) and adjusted to the indicated concentrations.

2.5. In vitro platelet aggregation

PRP from *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ mice were stimulated with the following agonists: different concentrations of U46619, ADP or TRAP4, as described before [14]. Platelet aggregation was measured by the turbidometric method using a model 700 aggregometry system (Chrono-Log Corporation, Havertown, PA). Each experiment was repeated at least 3 times and blood was pooled from at least three separate groups of eight mice.

2.6. Dense granule release

Platelets were prepared as described above (250 μL; 2.5 × 10^8/mL) before being placed into siliconized cuvettes and stirred for 5 min at 37 °C at 1200 rpm. The luciferase substrate/luciferase mixture (12.5 μL, Chrono-Log) was then added, followed by the addition of U46619, ADP or TRAP4.

2.7. Measurement of receptor-operated calcium entry

Intra-platelet calcium was measured using Fura-2-acetoxymethyl ester (Fura-2AM) as described [19]. Mice platelets (2.0 × 10^8/ml) were labeled with 12.5 μM Fura-2AM and 0.2% Pluronic F-127 in HEPES/Tyrode buffer (pH 7.4) for 45 min at 37 °C. After washing, the platelets were resuspended without apyrase to a concentration of 2.0 × 10^8/ml. Samples (1 ml) were added to siliconized cuvettes, recalcified with 0.7 mM $CaCl_2$, were stimulated with Thapsigargin (TG) 100 nM for 180 s and then stimulated with 1.5 μM U46619, 10 μM ADP or 80 μM TRAP4. Furthermore, platelet suspensions were incubated for 60 s with indomethacin (10 μM) when 1 μM ADP was the agonist, for 3 min with constant stirring. Fluorescence was analyzed by excitation at 340 nm and 380 nm and emission was measured at 509 nm using a model

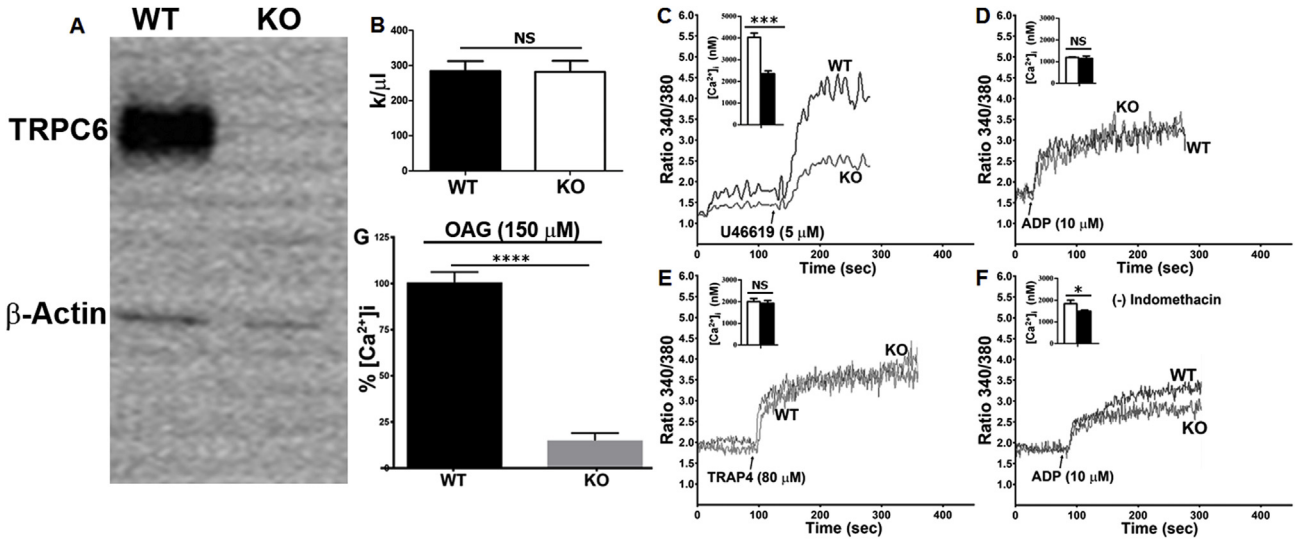

Fig. 1. TRPC6 deficiency does not affect platelet count but results in defective receptor-operated calcium entry is defective in *Trpc6*$^{-/-}$ platelets. (A) Platelet extracts (2×10^8/ml) were prepared from *Trpc6*$^{+/+}$ and the *Trpc6*$^{-/-}$ mice, and proteins (including β-Actin as loading control) were probed using Western Blot. (B) Platelet count was performed for the *Trpc6*$^{+/+}$ and the *Trpc6*$^{-/-}$ mice.
Trpc6$^{+/+}$ and *Trpc6*$^{-/-}$ washed platelets were loaded with Fura-2/AM and activated with either U46619 (5 μM; C; inset shows quantification of data expressed as mean $[Ca^{2+}]_i \pm$ SEM ($n = 3$)); ADP (10 μM; D; inset shows quantification of data expressed as mean $[Ca^{2+}]_i \pm$ SEM ($n = 3$)); TRAP4 (80 μM; E; inset shows quantification of data expressed as mean $[Ca^{2+}]_i \pm$ SEM ($n = 3$)), or ADP (10 μM; F inset shows quantification of data expressed as mean $[Ca^{2+}]_i \pm$ SEM ($n = 3$))) in the absence of indomethacin, before the maximum intracellular calcium levels were measured at the end of the measurement. (G) *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ washed platelets were loaded with Fura-2/AM and activated with OAG (150 μM). NS: Non-significant; *P < 0.05; ***P < 0.001; ****P < 0.0001, *t*-test. Each experiment was repeated 3 times, with blood pooled from three groups of 8–10 mice that were 8–10 weeks old. Data was analyzed using *t*-test.

LS50B Luminescence Spectrometer (PerkinElmer Instruments, Shelton, CT). The ratio of fura-2 emissions were calculated simultaneously using FL WinLab software and converted to $[Ca^{2+}]_i$, as described previously [19]. Briefly, Changes in 340/380 nm fura-2 fluorescence ratio were calibrated using Triton x-100 and EGTA in terms of $[Ca^{2+}]_i$ [20]. Ca^{2+} entry was estimated as the integral of the rise in $[Ca^{2+}]_i$ when the maximum point of the curve was attained, and compared to the maximum point observed of the trace after addition of the agonist, and applying the equation of Grynkiewicz to convert fura-2 ratios to $[Ca^{2+}]_i$ [21,22]. Of note, this experimental design (TG pre-stimulation) is essential for measuring ROCE, independent of "contamination" by calcium mobilization or SOCE, given the relativel lower magnitude of ROCE, compared to other means of intracellular calcium [23]. Hence, the GPCR agonist was added after TG would have "triggered" and "eliminated" contribution from (1) Ca^{2+} release from intracellular Ca^{2+} pools, (2) passive depletion of intracellular Ca^{2+} stores through SERCA inhibition, and (3) store operated Ca^{2+} entry (SOCE), with the exception of ROCE.

2.8. Flow cytometry

Flow cytometric analysis was carried out as discussed before [18,19,24,25]. Briefly, 5 μL of whole blood were added to tubes containing 95 μL HBS, and preincubated with anti-mouse CD16/CD32 Fc block (1μg/condition) for 15 min. Suspensions were centrifuged (1200 × *g*, 5 min) and the pellet resuspended in 100 μl PBS-Hepes Buffer Saline (HBS) with 1% BSA and 0.1% sodium azide. Platelets were then activated with U46619 (1.5 μM), ADP (10 μM) or TRAP4 (80 μM) for 2 min at room temperature. Controls were incubated with the same volume of the vehicle used for dilute the agonists. Some samples were incubated with indomethacin (10 μM) for 1 min before the addition of ADP. Platelets were incubated with FITC-conjugated anti-P-selectin or JON/A antibodies at room temperature for 30 min in the dark. The platelets were then diluted 2.5-fold with HEPES/Tyrode's buffer (pH 7.4). The samples were transferred to FACS-tubes and fluorescent intensities were measured using a BD Accuri C6 flow cytometer and analyzed using CFlow Plus (BD Biosciences, Franklin Lakes, NJ).

2.9. Clot retraction assay

The clot retraction assay was performed as we described before [19,25]. Briefly, blood was collected from the ventricle, followed by centrifugation at 237 × *g* for 10 min at RT to collect PRP, before platelets were pelleted at 483 × *g* for 10 min at RT. The pellets were resuspended in HEPES/Tyrode's buffer supplemented with 1 mM EGTA, 0.37 U/mL apyrase, and 10 ng/mL PGI_2. Washed platelets were isolated as discussed above. $CaCl_2$ was added extemporaneously at a final concentration of 1 mM, and 2 μl of erythrocytes were also added to enhance the contrast of the clot. First, a 10% (w/v) polyacrylamide cushion was polymerized at the bottom of the tubes to avoid clot adherence. Tubes were then rinsed extensively in distilled water. In platelet samples, fibrinogen (500 μg/mL) was added, and clot retraction was initiated by thrombin (0.4 U/mL), at room temperature. Pictures were taken at time intervals using a digital camera, and retraction quantified by digital processing and plotted as percentage of maximal retraction.

2.10. Platelet count

We counted platelets in whole blood obtained from knockout and wildtype mice using a HEMAVET® 950FS Multi-species Hematology System from Erba® Diagnostics (Miami Lakes, FL).

2.11. Statistical analysis

All experiments were performed at least three times, with blood pooled from at least 6–8 mice each time. Analysis of the data (student *t*-test) was performed using GraphPad PRISM statistical software (San Diego, CA). Significance was accepted at $P < 0.05$ (two-tailed P value), unless stated otherwise.

3. Results

3.1. TRPC6 deficiency does not affect platelet count

To discern the direct role of TRPC6 in platelets, we studied platelet function in mice with a global knockout of the *Trpc6* gene (*Trpc6*$^{-/-}$). The TRPC6 knockout strain showed no physical differences in comparison to their wild-type littermates, with both strains being a viable and healthy offspring. We first verified the genotype of the TRPC6 mice by analysis of genomic DNA, as we did previously [16] (data not shown), and by western blot (Fig. 1A). Furthermore, analysis of peripheral blood samples of *Trpc6*$^{-/-}$ mice and wild-type littermates showed that *Trpc6*$^{-/-}$ mice have normal platelet counts (Fig. 1B). These data suggest that TRPC6 deficiency does not affect thrombopoiesis.

3.2. Receptor-operated calcium entry is defective in Trpc6$^{-/-}$ platelets

Given that TRPC6 is a calcium channel, and that our previous pharmacological inhibitor findings showed that platelet function critically depends on increases in cytosolic calcium [Ca^{2+}]$_i$ concentration [19], we examined the direct role/effect of TRPC6 deletion on ROCE. It was found that [Ca^{2+}]$_i$/ROCE was impaired in response to the TPR agonist U46619 (5 μM) in the*Trpc6*$^{-/-}$ compared to their wild-type littermates (Fig. 1C; inset shows data quantification). On the other hand, there was no difference between the knockout and wild-type platelets, in response to separate agonists, namely ADP (10 μM; Fig. 1D; inset shows data quantification) or the selective protease-activated receptor 4 agonist, namely thrombin-receptor activating peptide 4 (TRAP4; 80 μM; Fig. 1E; inset shows data quantification). These results indicate that TRPC6 selectively mediates TPR-dependent ROCE, and suggests that it plays a critical role in platelet function. We also examined the ability of a documented TRPC6 agonist, the membrane permeable analogue of diacylglycerol (DAG), namely 1-oleoyl-2-acetyl-sn-glycerol (OAG) to modulates calcium levels. Our results revealed that TRPC6 deletion impaired OAG-triggered ROCE (Fig. 1G).

3.3. Deletion of TRPC6 alters agonist-induced platelet aggregation

We next examined if platelet function is affected by *Trpc6* gene deletion, by analyzing the platelet aggregation response, which is measured as a decrease in turbidity of a platelet-containing solution over time. Our studies demonstrated that the *Trpc6*$^{-/-}$ platelets exhibited a defect in platelet aggregation stimulated by the TPR agonist U46619 (1.5 μM or 5 μM) when compared to *Trpc6*$^{+/+}$ controls (Fig. 2A; data quantification is shown in Fig. 2K). Conversely, no significant differences in aggregation were seen when ADP was used (5 μM or 10 μM; Fig. 2B). Additional experiments were performed using TRAP4 (40 μM or 80 μM), but no significant difference in aggregation was observed between the *Trpc6*$^{-/-}$ and *Trpc6*$^{+/+}$ platelets (Fig. 2C). These results are consistent with the ROCE phenotype, and support the notion that the TRPC6 plays an important role in platelet activation in response to TPR, but not the ADP receptors or PAR4.

3.3.1. Dense and alpha granules release is defective in TRPC6-deficient platelets

In the next set of experiments, we sought to determine whether the TRPC6 deletion would exert inhibitory effects on platelet secretion. Hence, the *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ platelets were stimulated with various agonists and the ATP release (marker of dense granules) and p-selectin expression (marker of alpha granules) were measured. It was observed that ATP release and p-selectin expression were defective in the *Trpc6*$^{-/-}$ platelets when compared to *Trpc6*$^{+/+}$ platelets, when stimulated with U46619 (1.5 μM or 5 μM; Fig. 2E, I). On the other hand, there was no significant difference in ATP release or p-selectin expression when the knockout and wild-type platelets were stimulated with either ADP (5 μM or 10 μM; Fig. 2F, I) or TRAP4 (40 μM or 80 μM; Fig. 2G, I). These results indicate that TRPC6 plays an important role in platelet secretion, and that it does so in a manner that is selective to TPR stimulation.

3.3.2. Integrin αIIbβ3 activation is impaired in Trpc6$^{-/-}$ platelets

Integrin αIIbβ3 plays an important role in platelet aggregation in response to physiological agonists, and hence mediates thrombus formation [26,27]. We next investigated whether the impaired aggregation response would be associated with a commensurate inhibition of integrin αIIbβ3 activation. Indeed, our results indicated that the activation of integrin αIIbβ3 is significantly reduced (28% inhibition) in *Trpc6*$^{-/-}$ compared to *Trpc6*$^{+/+}$ platelets in response to U46619 (1.5 μM; Fig. 2J), but not with ADP (10 μM; Fig. 2J), or TRAP4 (80 μM; Fig. 2J).

3.4. Indomethacin reveals defects in ADP-induced platelet function in Trpc6$^{-/-}$ platelets

When the aforementioned experiments were repeated in the absence of indomethacin, a difference between ADP-stimulated ROCE, aggregation, secretion and integrin activation was revealed between the knockout and wild-type platelets (Figs. 1F, 2D and 2H, 2I and 2J). These results suggest that when ADP-induced platelet activation is examined under experimental conditions that allow for the involvement of TXA_2, a defect or difference between the TRPC6 deletion and wild-type platelets is “unmasked”.

3.5. Clot retraction is impaired in Trpc6$^{-/-}$ platelets

Platelet hyperactivity is associated with a number of pathophysiological conditions leading to abnormal clot formation, resulting in heart attacks and stroke. To investigate the possibility that TRPC6 may regulate the ability of platelets to generate contractile forces, we analyzed the effect of TRPC6 deletion on clot retraction. It was found that clot retraction was significantly delayed/inhibited in *Trpc6*$^{-/-}$ compared with *Trpc6*$^{+/+}$ platelets (Fig. 3). These results indicate that TRPC6 plays a key role in the generation of contractile forces, i.e., clot retraction.

3.6. TRPC6 expression in platelets increases with time

It was previously reported that the expression level of TRPC6 strongly depends on age of cells [28]. For example, TRPC6 expression was found to be upregulated by 6-fold with time, in fibroblasts [28]. It may be the case that the expression level of TRPC6 is age-dependent in megakaryocytes/platelets. This was indeed found to be the case, as the expression level of TRPC6 was found to increase with age in (wild-type) platelets (Fig. 4).

4. Discussion

There is evidence that calcium release from intracellular Ca^{2+} pools is transient and sometimes insufficient for full activation of platelets [29–31]. Under these conditions, Ca^{2+} influx through plasma membrane channels is essential for maintaining sustained Ca^{2+} signals and refilling the intracellular Ca^{2+} compartments. Moreover, TRPC6 channels have been described as Ca^{2+}-permeable cation channels responsible for receptor-operated/mediated Ca^{2+} entry (ROCE) in a variety of excitable and non-excitable cells, including platelets [32–34]. In fact, TRPC6 plays an important role in platelet function by regulating many different signaling pathways, including Ca^{2+} entry/ROCE [16,19,35–40]. To this end, while we have previously reported, using the TRPC6 deficient mice, that this channel plays an important role in hemostasis and thrombogenesis, whether it directly regulates platelet activation remains to be determined [16]. As for human platelets, using

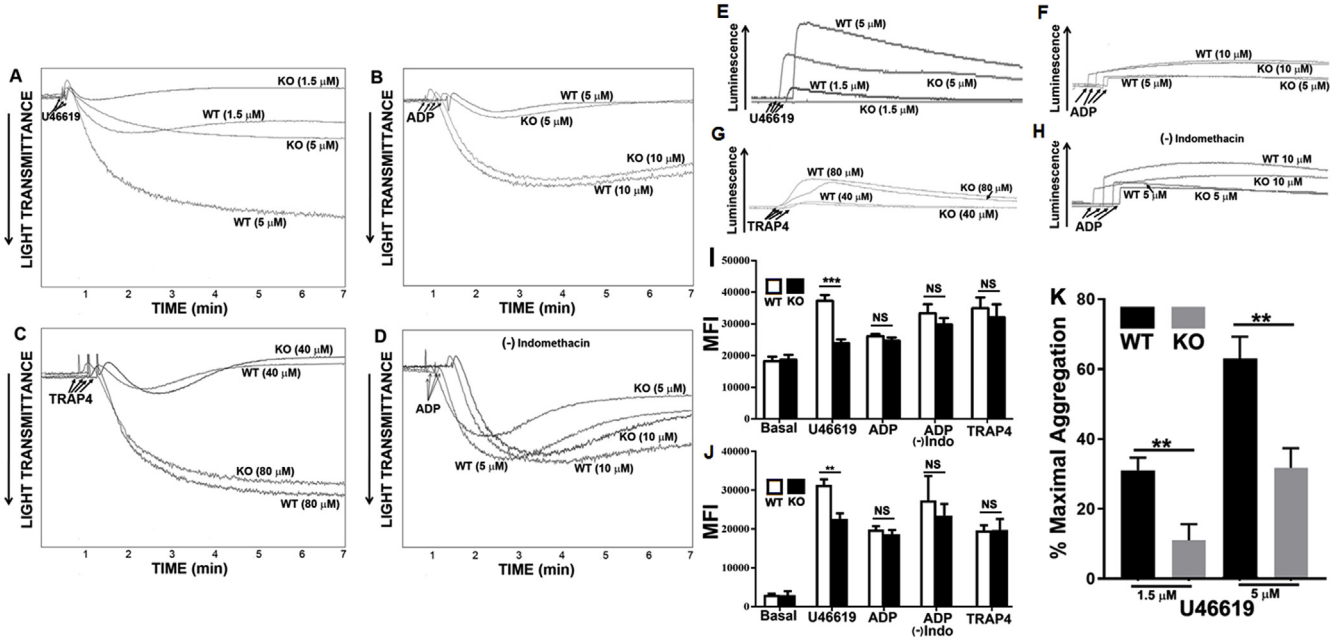

Fig. 2. Deletion of TRPC6 alters agonist-induced platelet aggregation, dense granule, alpha granules release and integrin αIIbβ3 activation. *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ mouse PRP containing 3×10^8 platelets/ml, were stimulated with either U46619 (1.5 μM and 5 μM; (A)), ADP (5 μM and 10 μM; (B)); or TRAP4 (40 μM and 80 μM; (C)) before the aggregation response was examined. (D) *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ mouse PRP containing 3×10^8 platelets/ml was stimulated with ADP (10 μM) in the absence of indomethacin, before the aggregation response was examined. Each experiment was repeated 3 times, with blood pooled from three groups of 8–10 mice. (E–G) 12.5 μL of luciferase luciferin was added to *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ platelets before they were stimulated with either U46619 (1.5 μM and 5 μM; (E)), ADP (5 μM and 10 μM; (F)); TRAP (40 μM and 80 μM; (G)). (H) *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ Platelets were stimulated with ADP (10 μM) in the absence of indomethacin. Release of ATP (for dense granule release) as a luminescence was measured by aggregometer. Each experiment was repeated 3 times, with blood pooled from three groups of 8–10 mice. *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ washed platelets were stimulated with either U46619 (5 μM), ADP (10 μM; in the presence or absence of indomethacin); or TRAP4 (80 μM) for 5 min. The reactions were stopped by fixing the platelets with 2% formaldehyde for 30 min at room temperature. (I) Platelets were incubated with FITC-conjugated anti-P-selectin antibody (for alpha granule), or (J) with FITC-conjugated anti-JON/A antibody (for integrin αIIbβ3). The fluorescent intensities were measured by flow cytometry, and the data were plotted as bar diagram; ***P $<$ 0.001; NS: Non-significant, *t*-test. (K) Quantification of U46619-induced aggregation in the *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ platelets; **P $<$ 0.01. Each experiment was repeated 3 times, with blood pooled from three groups of 8–10 mice that were 8–10 weeks old. Data was analyzed using *t*-test.

a TRPC6 inhibitor, we showed that TRPC6 regulates platelet activation and ROCE in a thromboxane receptor (TPR)-dependent manner [19]. Given the limitations of pharmacological inhibitors, including concerns of non-specificity, we sought to investigate if TRPC6 plays a direct role in platelets, using our deletion mice. In the present study, we have found that Trpc6$^{-/-}$ platelets exhibited an agonist/TPR-specific deficiency in ROCE, which resulted in impaired platelet activation and clot retraction.

Consistent with the notion that TRPC6 is a Ca^{2+}-permeable cation channel, and the one described as responsible for ROCE in platelets [33,36,41], our results did indeed show a significant reduction in ($[Ca^{2+}]_i$ in the *Trpc6*$^{-/-}$ platelets, which is commensurate with inhibition of ROCE. This data provides further evidence that TRPC6 modulates calcium homeostasis, in mouse platelets. As was the case with human platelets [19], the defect in ROCE was specific to agonists for the TPR pathway, as no effects were observed in response to ADP and the PAR4 agonist TRAP4, which supports the notion that TRPC6 regulates ROCE in a receptor specific manner. Also similar to our human platelet studies, our results have shown that *Trpc6*$^{-/-}$ platelets are defective in their aggregation, as well as dense and alpha granules release, which was again selective to the TPR pathway. The defect in secretion is consistent with a previous study that utilized an antibody that recognizes an extracellular amino acid sequence of TRPC6 [37]. Of note, there seems to be cell-specific differences with regard to regulation of agonist-induced function by TRPC6, as another study reported a vital role for the latter in thrombin-mediated endothelial cell contraction [23].

Our results also revealed that the *Trpc6*$^{-/-}$ platelets displayed impaired TPR-mediated integrin αIIbβ3 activation. These results indicate that the full activation of integrin αIIbβ3 downstream of TPR requires a threshold of intracellular calcium levels, some of which involves ROCE via TRPC6. This finding, and others, indicates that the platelet TRPC6 function is highly selective and depends on the activation of TPRs, but not PAR4 or the P2Y1 receptor. While this TPR specific phenotype is consistent with our human studies [19], some may consider them surprising given that the ADP and PAR4 pathways are also known to couple to Gq. Nonetheless, this finding provides evidence of the spatial or compartmentalized nature of TPR and/or its Gq, and the TRPC6-mediated regulation of calcium entry in platelets, as was previously documented with other cell systems [39,42].

Given the defect in its activation, and the fact that αIIbβ3 "outside-in" signaling is known to contribute to a number of platelet functional responses, including clot retraction [43], the latter was investigated in the TRPC6 deficient platelets. Indeed, clot retraction was defective in the *Trpc6*$^{-/-}$ platelets. This finding suggests that the αIIbβ3 defects observed in *Trpc6*$^{-/-}$ are mainly related to a compromised capacity to keep the $[Ca^{2+}]_i$ at suitable/threshold levels, as a result of impaired ROCE.

Collectively, these data serve, at least in part, as the underlying basis, and are consistent with/explain the phenotype we previously described in TRPC6 KO mice *in vivo*, i.e., namely impaired hemostasis and occlusive thrombus formation [16]. Moreover, our findings support the notion that anti-TRPC6 agents may have therapeutic implications in the context of cardiovascular disease. It is noteworthy that aside from expressing high levels of TRPC6, platelets are known to express low levels of TRPC1 [12,44], albeit the former is exclusively on the plasma membrane, whereas the latter is mostly in/on the intracellular membrane [12]. Moreover, the TRPC1 deletion mice/platelets displayed no

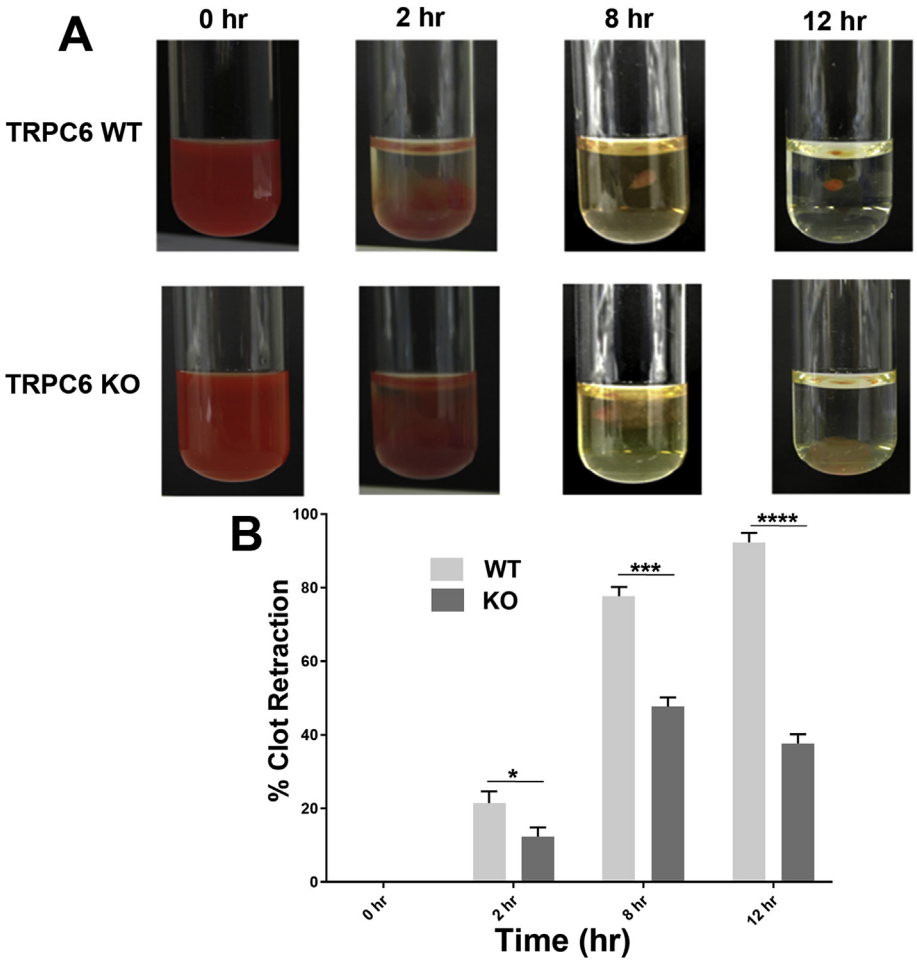

Fig. 3. Clot retraction is impaired in *Trpc6*$^{-/-}$ platelets. (A) *Trpc6*$^{+/+}$ and *Trpc6*$^{-/-}$ platelets were washed and resuspended at 1×10^8/mL in buffer (see "Methods") in the presence of 500 μg/mL fibrinogen, with erythrocytes included to enhance visualization. Fibrin clot formation was initiated by thrombin (0.4 U/mL) at 37 °C. Images represent time-frame of a retracting clot at the indicated time points. (B) Clot retraction quantification by digital processing and plotted as percentage of clot retraction; *P < 0.05; ***P < 0.001; ****P < 0.0001, *t*-test. This experiment was repeated 3 times, with blood pooled from three groups of 8–10 mice that were 8–10 weeks old.

apparent defects with regard to calcium homeostasis and platelet function [2].

Interestingly, when platelet function was assessed in the absence of the COX-1 inhibitor indomethacin, a "slight" defect/inhibition in platelet function in response to ADP was observed/unmasked. This finding is not necessarily surprising given the previously established role for COX-1/TXA_2/TPR in amplifying platelet activation and secretion via feedback mechanisms [23,45], as well as the role of phospholipase A_2 in ADP-induced platelet activation [46]. Thus, when the experimental conditions allow for the contribution of the synthesized TXA_2 to ADP-induced platelet function, a defect appears. Notably, the "unmasked" differences between the TRPC6 deletion and wildtype platelets did not always reach statistical significance, which we believe is attributed to the resolution of the assay we employed, but could also be dose dependent.

It is to be noted that another research group found no apparent "platelet" phenotype in the TRPC6 deficient mice/platelets, other than a defect in calcium entry triggered by diacylglycerol [3]. The discrepancies seem to derive-at least-from the following: 1. The genetic background of the mice, since ours were backcrossed 10 generations onto C57BL/6J, whereas it appears theirs were not (otherwise is not indicated); 2. differences in the age of the animals used in their study versus ours (4–12 weeks versus 8–10 weeks); and 3. differences in experimental conditions such as agonist concentrations, and the type of platelet preparation (platelet rich plasma (PRP) versus washed platelets, in our and their studies, respectively). The suggestion that age contributed to the differences is consistent with this findings that TRPC calcium dynamics and the expression of TRPC6 strongly depend on the growth stage of cells, or on their age [28]. To this end, it was shown that TRPC6 expression in fibroblasts is upregulated over time (up to 6-fold). As for platelets (megakaryocytes), we found that the expression level of TRPC6 is indeed age-dependent, and hence the four or six week old wild-type animals used by the other research group may not have expressed "much/enough" TRPC6 to reveal a phenotype/difference in comparison with the knockout mice.

It is also noteworthy that TRPC proteins have been reported to associate with different Ca^{2+}-handling proteins, including the type II inositol 1,4,5-trisphosphate receptor, the endoplasmic reticulum Ca^{2+} sensor STIM1 (STromal Interaction Molecule-1) or the Ca^{2+} permeable channel Orai1 [39]. The dynamic interaction of TRPC channels with the above mentioned proteins has been found to be important for both store-operated and capacitative Ca^{2+} entry, as well as for non-capacitative Ca^{2+} influx. The former is a major mechanism for Ca^{2+} entry in human platelets. This mechanism, activated by a reduction in the concentration of free Ca^{2+} in the intracellular stores, results in the formation of signaling complexes involving STIM proteins, Orai1, Orai2, TRPC1 and TRPC6 [47]. There is a growing body of evidence supporting that Ca^{2+} signaling dysfunction plays an important role in the pathogenesis of several platelet-linked disorders [6]. Abnormal Ca^{2+} signals in response to physiological agonists have also been associated with platelet hyperactivity, thus suggesting a role for Ca^{2+} handling proteins, including TRPs, in the pathogenesis of coagulation defects.

In summary, these data demonstrate that TRPC6 plays a key role in intracellular calcium changes, namely, the regulation of TPR-mediated ROCE. In turn, TRPC6 modulates platelet aggregation, secretion, integrin activation and clot retraction. These data indicate that TRPC6 may be a potential therapeutic target for managing thrombotic

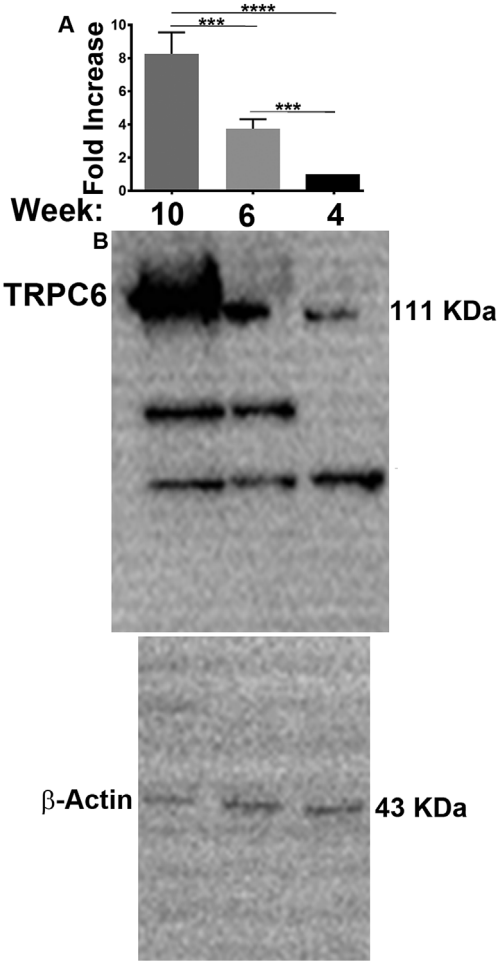

Fig. 4. TRPC6 expression increases with age. Platelet extracts (2×10^8/ml) from *Trpc6*$^{+/+}$ mice that are 4, 6 or 10 weeks-old were prepared, and the TRPC6 protein (and β-Actin as loading control) was probed using Western Blot. (A) Quantification of protein expression determined via Western blot; ***P $<$ 0.001; ****P $<$ 0.001, *t*-test. (B) Western blot data showing expression of TRPC6 in an age-dependent manner, along with β-Actin as loading control. This experiment was repeated 3 times, with blood pooled from three groups of 5–6 mice, at the specified age.

disorders.

Disclosures

None.

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References

[1] D. Blockmans, H. Deckmyn, J. Vermylen, Platelet activation, Blood Rev. 9 (1995) 143–156.
[2] D. Varga-Szabo, K.S. Authi, A. Braun, M. Bender, A. Ambily, S.R. Hassock, T. Gudermann, A. Dietrich, B. Nieswandt, Store-operated Ca(2+) entry in platelets occurs independently of transient receptor potential (TRP) C1, Pflüg. Arch. 457 (2008) 377–387.
[3] G. Ramanathan, S. Gupta, I. Thielmann, I. Pleines, D. Varga-Szabo, F. May, C. Mannhalter, A. Dietrich, B. Nieswandt, A. Braun, Defective diacylglycerol-induced Ca2+ entry but normal agonist-induced activation responses in TRPC6-deficient mouse platelets, J. Thromb. Haemost. 10 (2012) 419–429.
[4] D. Varga-Szabo, A. Braun, B. Nieswandt, Calcium signaling in platelets, J. Thromb. Haemost. 7 (2009) 1057–1066.
[5] Y. Zhang, E. Pertens, L.J. Janssen, 8-isoprostaglandin E(2) activates Ca(2+)-dependent K(+) current via cyclic AMP signaling pathway in murine renal artery, Eur. J. Pharmacol. 520 (2005) 22–28.
[6] M.P. Winn, P.J. Conlon, K.L. Lynn, M.K. Farrington, T. Creazzo, A.F. Hawkins, N. Daskalakis, S.Y. Kwan, S. Ebersviller, J.L. Burchette, M.A. Pericak-Vance, D.N. Howell, J.M. Vance, P.B. Rosenberg, A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis, Science 308 (2005) 1801–1804.
[7] A. Braun, D. Varga-Szabo, C. Kleinschnitz, I. Pleines, M. Bender, M. Austinat, M. Bosl, G. Stoll, B. Nieswandt, Orai1 (CRACM1) is the platelet SOC channel and essential for pathological thrombus formation, Blood 113 (2009) 2056–2063.
[8] K. Gilio, R. van Kruchten, A. Braun, A. Berna-Erro, M.A. Feijge, D. Stegner, P.E. van der Meijden, M.J. Kuijpers, D. Varga-Szabo, J.W. Heemskerk, B. Nieswandt, Roles of platelet STIM1 and Orai1 in glycoprotein VI- and thrombin-dependent procoagulant activity and thrombus formation, J. Biol. Chem. 285 (2010) 23629–23638.
[9] M. Estacion, W.G. Sinkins, S.W. Jones, M.A. Applegate, W.P. Schilling, Human TRPC6 expressed in HEK 293 cells forms non-selective cation channels with limited Ca2+ permeability, J. Physiol. 572 (2006) 359–377.
[10] A.P. Albert, Activation of TRPC6 channel proteins: evidence for an essential role of phosphorylation, J. Physiol. 561 (2004) 354.
[11] S.L. Brownlow, S.O. Sage, Transient receptor potential protein subunit assembly and membrane distribution in human platelets, Thromb. Haemost. 94 (2005) 839–845.
[12] S.R. Hassock, M.X. Zhu, C. Trost, V. Flockerzi, K.S. Authi, Expression and role of TRPC proteins in human platelets: evidence that TRPC6 forms the store-independent calcium entry channel, Blood 100 (2002) 2801–2811.
[13] K.S. Authi, TRP channels in platelet function, Handb. Exp. Pharmacol. (2007) 425–443.
[14] A. Gamberucci, E. Giurisato, P. Pizzo, M. Tassi, R. Giunti, D.P. McIntosh, A. Benedetti, Diacylglycerol activates the influx of extracellular cations in T-lymphocytes independently of intracellular calcium-store depletion and possibly involving endogenous TRP6 gene products, Biochem. J. 364 (2002) 245–254.
[15] T. Hofmann, A.G. Obukhov, M. Schaefer, C. Harteneck, T. Gudermann, G. Schultz, Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol, Nature 397 (1999) 259–263.
[16] E.V. Paez Espinosa, J.P. Murad, H.J. Ting, F.T. Khasawneh, Mouse transient receptor potential channel 6: role in hemostasis and thrombogenesis, Biochem. Biophys. Res. Commun. 417 (2012) 853–856.
[17] A. Dietrich, Y.S.M. Mederos, M. Gollasch, V. Gross, U. Storch, G. Dubrovska, M. Obst, E. Yildirim, B. Salanova, H. Kalwa, K. Essin, O. Pinkenburg, F.C. Luft, T. Gudermann, L. Birnbaumer, Increased vascular smooth muscle contractility in TRPC6-/- mice, Mol. Cell. Biol. 25 (2005) 6980–6989.
[18] N.R. Hensch, Z.A. Karim, K.M. Druey, M.G. Tansey, F.T. Khasawneh, RGS10 negatively regulates platelet activation and thrombogenesis, PLoS One 11 (2016) e0165984.
[19] H.P. Vemana, Z.A. Karim, C. Conlon, F.T. Khasawneh, A critical role for the transient receptor potential channel type 6 in human platelet activation, PLoS One 10 (2015) e0125764.
[20] J.J. Lopez, P.C. Redondo, G.M. Salido, J.A. Pariente, J.A. Rosado, N,N,N',N'-tetrakis (2-pyridylmethyl)ethylenediamine induces apoptosis through the activation of caspases-3 and -8 in human platelets. A role for endoplasmic reticulum stress, J. Thromb. Haemost. : JTH 7 (2009) 992–999.
[21] M.J. Mason, M.P. Mahaut-Smith, Measurement and manipulation of intracellular Ca2+ in single platelets and megakaryocytes, Methods Mol. Biol. 273 (2004) 251–276 (Clifton, N.J.).
[22] G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca2+ indicators with greatly improved fluorescence properties, J. Biol. Chem. 260 (1985) 3440–3450.
[23] I. Singh, N. Knezevic, G.U. Ahmmed, V. Kini, A.B. Malik, D. Mehta, Galphaq-TRPC6-mediated Ca2+ entry induces RhoA activation and resultant endothelial cell shape change in response to thrombin, J. Biol. Chem. 282 (2007) 7833–7843.
[24] N.R. Hensch, Z.A. Karim, J. Pineda, N. Mercado, F.Z. Alshbool, F.T. Khasawneh, P2Y12 antibody inhibits platelet activity and protects against thrombogenesis, Biochem. Biophys. Res. Commun. 493 (2017) 1069–1074.
[25] Z.A. Karim, H.P. Vemana, F.T. Khasawneh, MALT1-ubiquitination triggers non-genomic NF-kappaB/IKK signaling upon platelet activation, PLoS One 10 (2015) e0119363.
[26] P. Hook, R.I. Litvinov, O.V. Kim, S. Xu, Z. Xu, J.S. Bennett, M.S. Alber, J.W. Weisel, Strong binding of platelet integrin alphaIIbbeta3 to fibrin clots: potential target to destabilize thrombi, Sci. Rep. 7 (2017) 13001.
[27] H. Nguyen, K. Huynh, V.R. Stoldt, Shear-dependent fibrillogenesis of fibronectin: impact of platelet integrins and actin cytoskeleton, Biochem. Biophys. Res. Commun. 497 (2018) 797–803.
[28] W.H. Almirza, P.H. Peters, E.J. van Zoelen, A.P. Theuvenet, Role of Trpc channels, Stim1 and Orai1 in PGF(2alpha)-induced calcium signaling in NRK fibroblasts, Cell Calcium 51 (2012) 12–21.
[29] B. Brune, F. von Appen, V. Ullrich, Receptor occupancy regulates Ca2+ entry and intracellular Ca2+ redistribution in activated human platelets, Biochem. J. 304 (Pt 3) (1994) 993–999.
[30] Y. Liao, J. Abramowitz, L. Birnbaumer, The TRPC family of TRP channels: roles inferred (mostly) from knockout mice and relationship to ORAI proteins, Handb. Exp. Pharmacol. 223 (2014) 1055–1075.
[31] B. Nilius, V. Flockerzi, Mammalian transient receptor potential (TRP) cation channels, Preface, Handb Exp Pharmacol 223 (2014) (v - vi).

[32] S. Jung, R. Strotmann, G. Schultz, T.D. Plant, TRPC6 is a candidate channel involved in receptor-stimulated cation currents in A7r5 smooth muscle cells, Am. J. Physiol. Cell Physiol. 282 (2002) C347–C359.
[33] Y. Liao, N.W. Plummer, M.D. George, J. Abramowitz, M.X. Zhu, L. Birnbaumer, A role for Orai in TRPC-mediated Ca2+ entry suggests that a TRPC:Orai complex may mediate store and receptor operated Ca2+ entry, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 3202–3206.
[34] S.O. Sage, S.L. Brownlow, J.A. Rosado, TRP channels and calcium entry in human platelets, Blood 100 (2002) 4245–4246 author reply 4246-4247.
[35] A. Berna-Erro, I. Jardin, T. Smani, J.A. Rosado, Regulation of platelet function by Orai, STIM and TRP, Adv. Exp. Med. Biol. 898 (2016) 157–181.
[36] Z. Ilkan, J.R. Wright, A.H. Goodall, J.M. Gibbins, C.I. Jones, M.P. Mahaut-Smith, Evidence for shear-mediated Ca(2+) entry through mechanosensitive cation channels in human platelets and a megakaryocytic cell line, J. Biol. Chem. 292 (2017) 9204–9217.
[37] E. Lopez, N. Bermejo, A. Berna-Erro, N. Alonso, G.M. Salido, P.C. Redondo, J.A. Rosado, Relationship between calcium mobilization and platelet alpha- and delta-granule secretion. A role for TRPC6 in thrombin-evoked delta-granule exocytosis, Arch. Biochem. Biophys. 585 (2015) 75–81.
[38] E. Lopez, A. Berna-Erro, G.M. Salido, J.A. Rosado, P.C. Redondo, FKBP25 and FKBP38 regulate non-capacitative calcium entry through TRPC6, Biochim. Biophys. Acta 1853 (2015) 2684–2696.
[39] A.B. Parekh, J.W. Putney Jr., Store-operated calcium channels, Physiol. Rev. 85 (2005) 757–810.
[40] G. Ramanathan, C. Mannhalter, Increased expression of transient receptor potential canonical 6 (TRPC6) in differentiating human megakaryocytes, Cell Biol. Int. 40 (2016) 223–231.
[41] A. Bouron, S. Chauvet, S. Dryer, J.A. Rosado, Second messenger-operated calcium entry through TRPC6, Adv. Exp. Med. Biol. 898 (2016) 201–249.
[42] D. Krizaj, Compartmentalization of calcium entry pathways in mouse rods, Eur. J. Neurosci. 22 (2005) 3292–3296.
[43] Z. Li, M.K. Delaney, K.A. O'Brien, X. Du, Signaling during platelet adhesion and activation, Arterioscler. Thromb. Vasc. Biol. 30 (2010) 2341–2349.
[44] R.N. Carter, G. Tolhurst, G. Walmsley, M. Vizuete-Forster, N. Miller, M.P. Mahaut-Smith, Molecular and electrophysiological characterization of transient receptor potential ion channels in the primary murine megakaryocyte, J. Physiol. 576 (2006) 151–162.
[45] P.T. Dancs, E. Ruisanchez, A. Balogh, C.R. Panta, Z. Miklos, R.M. Nusing, J. Aoki, J. Chun, S. Offermanns, G. Tigyi, Z. Benyo, LPA1 receptor-mediated thromboxane A2 release is responsible for lysophosphatidic acid-induced vascular smooth muscle contraction, FASEB J. 31 (2017) 1547–1555.
[46] E. Yoda, K. Rai, M. Ogawa, Y. Takakura, H. Kuwata, H. Suzuki, Y. Nakatani, M. Murakami, S. Hara, Group VIB calcium-independent phospholipase A2 (iPLA2gamma) regulates platelet activation, hemostasis and thrombosis in mice, PLoS One 9 (2014) e109409.
[47] H.L. Ong, K.T. Cheng, X. Liu, B.C. Bandyopadhyay, B.C. Paria, J. Soboloff, B. Pani, Y. Gwack, S. Srikanth, B.B. Singh, D.L. Gill, I.S. Ambudkar, Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components, J. Biol. Chem. 282 (2007) 9105–9116.