Cav\(_{3.2}\) T-type calcium channel regulates mouse platelet activation and arterial thrombosis

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Abstract

**Background:** Cav\(_{3.2}\) is a T-type calcium channel that causes low-threshold exocytosis. T-type calcium channel blockers reduce platelet granule exocytosis and aggregation. However, studies of the T-type calcium channel in platelets are lacking.

**Objective:** To examine the expression and role of Cav\(_{3.2}\) in platelet function.

**Methods:** Global Cav\(_{3.2}\)\(^{-/-}\) and platelet-specific Cav\(_{3.2}\)\(^{-/-}\) mice and littermate controls were used for this study. Western blot analysis was used to detect the presence of Cav\(_{3.2}\) and activation of the calcium-responsive protein extracellular signal-regulated kinase (ERK). Fura-2 dye was used to assess platelet calcium. Flow cytometry and light transmission aggregometry were used to evaluate platelet activation markers and aggregation, respectively. FeCl\(_3\)-induced thrombosis and a microfluidic flow device were used to assess in vivo and ex vivo thrombosis, respectively.

**Results:** Cav\(_{3.2}\) was expressed in mouse platelets. As compared with wild-type controls, Cav\(_{3.2}\)\(^{-/-}\) mouse platelets showed reduced calcium influx. Similarly, treatment with the T-type calcium channel inhibitor Ni\(^{2+}\) decreased the calcium influx in wild-type platelets. As compared with controls, both Cav\(_{3.2}\)\(^{-/-}\) and Ni\(^{2+}\)-treated wild-type platelets showed reduced activation of ERK. ATP release, P-selectin exposure, and \(\alpha_{IIb}\beta_{3}\) activation were reduced in Cav\(_{3.2}\)\(^{-/-}\) and Ni\(^{2+}\)-treated wild-type platelets, as was platelet aggregation. On in vivo and ex vivo thrombosis assay, Cav3.2 deletion caused delayed thrombus formation. However, tail bleeding assay showed intact hemostasis.

**Conclusion:** These results suggest that Cav\(_{3.2}\) is required for the optimal activation of platelets.

**KEYWORDS**
calcium, platelet, platelet aggregation, thrombosis, voltage-gated
1 | INTRODUCTION

Vascular injury triggers platelet adhesion and activation, leukocyte recruitment, release of growth factors, vascular smooth muscle cell proliferation and migration, and scarring of the vessel.1–5 Platelets play an important role in hemostasis and vessel integrity.6,7 However, inappropriate activation of platelets causes life-threatening arterial thrombosis.8

During platelet activation, increased level of intracellular Ca^{2+} ([Ca^{2+}]_{i}) mediates signal transduction, which leads to platelet activation and aggregation.9 Ca^{2+} release from internal stores and Ca^{2+} influx from the extracellular space results in increased [Ca^{2+}]_{i} level. In addition to the known store-operated calcium entry (SOCE) and receptor-operated calcium channels, some evidence supports the possible existence of other calcium channels.10–13 Efonidipine, a dual T- and L-type calcium channel blocker, has a strong antiplatelet effect.10 Efonidipine improves vascular endothelial function14 and reduces activation markers in platelets and monocytes in hypertensive patients.10

T-type calcium channels are voltage-gated calcium channels that activate at lower membrane potentials (approximately −70 to −60 mV) and mediate transient calcium currents. Ca_{v3.1}, Ca_{v3.2}, and Ca_{v3.3} are the three different isoforms present in mammals.15,16 Although predominantly expressed in excitable cells,17 Ca_{v3.1} and Ca_{v3.2} are found in several nonexcitable cells.18–21 T-type calcium channels exert their function tissue-specifically. Ca_{v3.2} mediates exocytosis in rat chromaffin cells,22,23 cardiac hypertrophy in response to pressure overload,24 and tracheal chondrogenesis.25 Similarly, Ca_{v3.1} regulates vascular smooth muscle cell proliferation during neointimal formation26 and calcium-dependent von Willebrand factor release from lung endothelial cells.27 Antiplatelet activity of T-type calcium channel blockers10 and evidence of T-type calcium channel-mediated exocytosis in nonexcitable cells indicates the possibility of involvement of these calcium channels in platelets.28,29 However, study of the presence and role of T-type calcium channels in platelets is lacking.

The current study focused on the role of Ca_{v3.2} T-type calcium channel in platelet activity and arterial thrombosis. Ca_{v3.2} is a transmembrane calcium channel. Therefore, we investigated the effect of Ca_{v3.2} deletion and treatment with Ni^{2+} (an inhibitor of the Ca_{v3.2} T-type calcium channel) in the change in [Ca^{2+}]_{i} level in platelets and downstream extracellular signal-regulated kinase (ERK) activation. Calcium-activated ERK is associated with platelet granule release and integrin activation, so we assessed platelet granule release by measuring adenosine triphosphate (ATP) release, P-selectin exposure and α_{IIb}β_{3} integrin activation. We further used a platelet functional study, measuring platelet aggregation of Ca_{v3.2}^{-/-} and Ni^{2+}-treated platelets. Next, we used FeCl_{3}-induced thrombosis to study arterial thrombosis in mice with knockout of Ca_{v3.2} (global and platelet-specific). This model mimics the endothelial damage and extracellular matrix exposure that mediates thrombus formation. To further consolidate our in vivo thrombosis findings, we used an ex vivo thrombosis assay with a microfluidic flow chamber device that simulates the blood flow, vessel wall injury, and thrombus growth.

2 | METHODS

2.1 | Mice

All conducted research conform to the appropriate US National Institutes of Health guidelines and those of the Institutional Animal Care and Utilization Committee, Academia Sinica and Far Eastern Memorial Hospital (Taipei). Adult male and female C57BL/6J mice 8–16 weeks old were used as controls. Global Ca_{v3.2}^{-/-} mice were generated as described.28 To generate platelet-specific Ca_{v3.2} conditional knockout (Ca_{v3.2}^{fl/fl}) mice, we crossbred platelet factor 4 (pf4^{cre/+})29,30 and Ca_{v3.2}^{fl/fl} mice (detailed methods for generating flox mice are described in Supplementary Materials). Mice with the genotype pf4^{cre/+}; Ca_{v3.2}^{fl/fl} were defined as Ca_{v3.2}^{2R+/-} mice and those with the genotype pf4^{cre/+}; Ca_{v3.2}^{2R+/+} were littermate controls. We used C57BL/6J wild-type control mice that were age- and sex-matched to global Ca_{v3.2}^{-/-} mice and Ca_{v3.2}^{2R+/-} mice matched to platelet-specific Ca_{v3.2}^{-/-} mice (Ca_{v3.2}^{2R+/-}). The primer sets (1+2; forward: 5’-aataccagcttgcatcgt-3’ and reverse: 5’-gtatactggagggacatgg-3’) were used for G protein-coupled receptor to confirm the Ca_{v3.2}^{2R+/-} and Ca_{v3.2}^{2R+/-} conditional knockout.

2.2 | Measurement of intracellular calcium ([Ca^{2+}]_{i})

Fura-2 (10 μM) was added to 7.5 × 10^{8} cells/ml platelets in Tyrode’s albumin buffer and incubated at 37°C for 40 min. The platelets were then washed three times. Finally, fura-2-loaded platelets were adjusted to 7 × 10^{8} cells/ml and CaCl_{2} (2 mM) was added as required. For global calcium concentration studies, thrombin and calcium were added together, and for calcium influx study, calcium was added 2 min after thrombin stimulation. For adenosine diphosphate (ADP)-induced rescue studies, ADP (0.02 μM) was added immediately after the addition of thrombin. Platelets were activated with thrombin/ADP, then Triton X-100 (0.1%), and EGTA (8 mM), and fluorescence intensity was measured by spectrofluorometry (FP8500, JASCO). The platelet [Ca^{2+}]_{i} level was calculated as described.31 The following formula was used to calculate calcium concentration: [Ca^{2+}]_{i} = K_{D} X[(R_{max}-R_{min})/(R_{max} -R)]X(5F_{2} /S_{b}), where K_{D} =
the dissociation constant of the dye for Ca$^{2+}$ at the chosen experimental condition ($K_D = 224$ nM at our experimental conditions); $R$ = the ratio of the fluorescence intensities at the two wavelengths (340/380); $R_{\text{min}}$ = the ratio value obtained after the addition of EGTA 8 mM; $R_{\text{max}}$ = the ratio value obtained after the addition of Triton X-100 0.1%; $S_f = 2$ = the maximum fluorescence intensity obtained at 380 nM; and $S_b = 2$ = the minimum fluorescence intensity obtained at 380 nM.

2.3 | Western blot analysis

Western blot analysis was performed as described. In brief, platelets were activated with agonists at 37°C in an aggregometer, and an equal volume of ice-cold 2X lysis buffer (Tris/HCl 100 mM, pH 7.4, NaCl 400 mM, MgCl$_2$ 5 mM, Nonidet P-40 2%, glycerol 20%, and complete protease inhibitor cocktail lacking EDTA) was added after 3 min. Protein lysates were run through the sodium dodecyl sulfate–polyacrylamide gel electrophoresis, then transferred onto PVDF membranes. The proteins were probed with phosphorylated ERK (pERK), tERK, and β-actin antibodies. Western blot images were taken by using LAS-4000 mini (Fujifilm) and images were analyzed and quantified by using ImageJ.

We transiently expressed Ca$_{\text{v}3.2}$ (human and mouse clone) and Ca$_{\text{v}3.1}$ (human clone) in HEK 293 cells. We used total protein lysates of these cells for control experiments and the membrane protein-enriched fraction of mouse platelets and testes for sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes and anti-Ca$_{\text{v}3.2}$ antibody C1868 (Sigma) was used to detect signals.

2.4 | Electron microscopy

Platelets were fixed with glutaraldehyde (2.5%) in a phosphate buffer (0.1 M, pH 7.4) for 1 h at room temperature. After fixation, the sample was washed three times with phosphate buffer and processed as described. Images were obtained by using a transmission electron microscope (FEI Tecnai G2 F20 S-TWIN).

2.5 | Statistical analysis

Statistical analysis was performed with Sigmaplot and GraphPad Prism 6. Unpaired Student t-tests, Mann-Whitney U test (nonparametric), one-way repeated measures analysis of variance (ANOVA) with Holm-Sidak post hoc test or one-way ANOVA with Tukey post hoc test were used to assess statistical significance. For all experiments, $p < .05$ was considered statistically significant. Additional methods and materials are in Supplementary files.

2.6 | Data sharing statement

For original data, please contact Chien-Change Chen at ccchen@ibms.sinica.edu.tw.

3 | RESULTS

3.1 | Expression of Ca$_{\text{v}3.2}$ T type calcium channel in mouse platelets

Our reverse transcriptase-polymerase chain reaction results from CD41-positive cells (megakaryocytes) demonstrated that Ca$_{\text{v}3.2}$ was expressed in mouse megakaryocytes (Figure 1A). To further confirm the expression of Ca$_{\text{v}3.2}$ in platelets, we used western blot analysis.

We tested several commercially available antibodies to detect Cav3.2 in platelets and finally chose anti-Ca$_{\text{v}3.2}$ antibody C1868 (Sigma) for our experiments. We first tested the specificity of the antibodies by

![Figure 1](image-url)
using Ca$_3$2.3 or Ca$_3$3.1 transiently expressed in HEK 293 cells. The antibody could detect Ca$_3$3.2 but not Ca$_3$3.1 expressed in HEK 293 cells (Figure 1B). Similarly, Ca$_3$3.2 could be detected in wild-type mouse platelets and testes but not in Ca$_3$3.2$^{-/-}$ controls (Figure 1C).

Next, we performed a complete blood cell count. Ca$_3$3.2$^{-/-}$ or Ca$_3$3.2$^{fl/fl}$ (global or platelet specific) mice and controls (Table 1) did not differ in counts. Similarly, we found no significant difference in granulation or morphology in Ca$_3$3.2$^{-/-}$ platelets (Figure 1D).

### 3.2 | Activation-induced change in [Ca$^{2+}$]$_i$ level was decreased in Ca$_3$3.2$^{-/-}$ and Ni$^{2+}$-treated platelets

Stimulation elevates [Ca$^{2+}$]$_i$ level in platelets. To study the role of Ca$_3$3.2 in change in platelet [Ca$^{2+}$]$_i$ concentration, we used fura-2-loaded platelets to access the global [Ca$^{2+}$]$_i$ concentration, calcium release from internal stores and calcium entry. Ca$_3$3.2$^{-/-}$ platelets showed reduced global change in calcium concentration in response to thrombin (451 ± 15 nM for Ca$_3$3.2$^{-/-}$ vs. 530 ± 12 nM for controls, p = .015; Figure 2A). All calcium concentration values stated are peak values.

To differentiate between calcium release or calcium entry defects, we activated platelets in the presence of EGTA (1 mM) for 2 min to induce calcium release from internal stores, then CaCl$_2$ (2 mM) was added to induce calcium influx. The calcium release from internal stores was minimal but similar in both Ca$_3$3.2$^{-/-}$ and wild-type platelets. However, Ca$_3$3.2$^{-/-}$ platelets showed decreased calcium influx after the addition of CaCl$_2$ (2 mM) (431 ± 13 nM for Ca$_3$3.2$^{-/-}$ vs. 499 ± 17 nM for controls, p = .01; Figure 2B). To rule out the effect of released ATP and ADP, calcium influx was measured in the presence of apyrase (5 U/ml). The findings confirm that the calcium influx defect in Ca$_3$3.2$^{-/-}$ platelets (443.3 ± 10.9 nM for Ca$_3$3.2$^{-/-}$ vs. 513 ± 14.2 nM for controls, p = .02; Figure 2C) was independent of ADP release.

Store-operated calcium entry is important for elevating [Ca$^{2+}$]$_i$ level. To evaluate SOCE, we assessed calcium influx induced by thapsigargin (100 nM). Calcium influx mediated by thapsigargin was comparable in Ca$_3$3.2$^{-/-}$ and wild-type controls (931.2 ± 50.8 vs. 897 ± 53.1, p = .9; Figure S1A). Thus, calcium influx defect in Ca$_3$3.2$^{-/-}$ platelets was not attributed to SOCE.

Ni$^{2+}$ at low concentrations specifically inhibits Ca$_3$3.2. We used NiCl$_2$ to assess the effect of Ni$^{2+}$ on change in platelet [Ca$^{2+}$]$_i$ level. Ni$^{2+}$ treatment (30 μM) reduced the global calcium concentration in response to thrombin (325 ± 16.5 for Ni$^{2+}$-treated platelets vs. 397.7 ± 14.6 for vehicle controls, p = .002; Figure 2D). Similarly, calcium influx but not calcium release was reduced in Ni$^{2+}$-treated platelets (280.1 ± 10.7 nM for Ni$^{2+}$-treated platelets vs. 337.5 ± 19 nM for vehicle controls, p = .01; Figure 2E). Ni$^{2+}$ reduced both global calcium concentration and calcium influx. Decreased calcium influx induced by Ni$^{2+}$ was not affected by apyrase (224.9 ± 31.3 nM for Ni$^{2+}$-treated platelets vs. 266.2 ± 24.8 nM for vehicle control, p = .04; Figure 2F). Ni$^{2+}$ treatment had no effect on calcium influx mediated by thapsigargin in wild-type platelets (Figure S1B). These results suggest that Ca$_3$3.2 plays a role in calcium influx.

### 3.3 | Deletion of Ca$_3$3.2 or application of its inhibitor (Ni$^{2+}$) reduced phosphorylation of ERK in platelets during activation

Calcium-mediated phosphorylation of ERK mediates platelet activity. Moreover, Ca$_3$3.2-dependent activation of ERK in the

|                 | WT         | Ca$_3$3.2$^{-/-}$ | Ca$_3$3.2$^{fl/fl}$ | Ca$_3$3.2$^{pt/pt}$ |
|-----------------|------------|------------------|--------------------|---------------------|
| Hb (g/dl)       | 15.01 ± 0.18 | 14.27 ± 0.34     | 15.92 ± 0.33       | 14.5 ± 0.53         |
| WBC count (10$^3$/μl) | 8.97 ± 1.00 | 9.4 ± 1.03       | 9.58 ± 0.94        | 9.93 ± 0.72         |
| RBC count (10$^7$/μl) | 9.82 ± 0.18 | 9.06 ± 0.23      | 9.97 ± 0.25        | 9.77 ± 0.37         |
| Hematocrit (%)  | 50.6 ± 0.79  | 47.2 ± 1.17      | 48.44 ± 1.81       | 51.2 ± 1.87         |
| Platelet count (10$^5$/μl) | 819 ± 60.62 | 760.28 ± 64.54  | 725.8 ± 38.6       | 768.2 ± 46.9        |
| MPV (FL)        | 7.54 ± 0.22  | 7.60 ± 0.21      | 7.14 ± 0.04        | 7.42 ± 0.03         |

Note: An automated hematology cell counter was used for complete blood cell counts. Data are mean ± SEM.

N = 7 (WT and Ca$_3$3.2$^{-/-}$) and N = 5 (Ca$_3$3.2$^{fl/fl}$ and Ca$_3$3.2$^{pt/pt}$).

Hematologic parameter values did not significantly differ among the four groups.

Hb, hemoglobin; MPV, mean platelet volume; RBC, red blood cell; WBC, white blood cell.
paraventricular thalamus regulates chronic pain. Our western blot results demonstrated that ERK phosphorylation induced by thrombin was significantly reduced in Ca$_{3.2}^{-/-}$ platelets ($p = .01$; Figure 3A,B).

Similarly, ERK phosphorylation was significantly reduced in Ni$^{2+}$-treated platelets ($p = .04$; Figure 3C,D). Ni$^{2+}$ did not further decrease the pERK level in Ca$_{3.2}^{-/-}$ platelets (Figure 3E,F). Thus, deletion of Cav3.2 or its inhibitor reduced ERK activation.

### 3.4 Granule secretion and activation of integrin $\alpha_{\text{IIb}}\beta_3$ is impaired in Ca$_{3.2}^{-/-}$ platelets

Calcium mediates platelet secretion and $\alpha_{\text{IIb}}\beta_3$ activation via ERK activation. ATP release induced by collagen ($62.7 \pm 8.5$ picomole/10$^7$ cells for Ca$_{3.2}^{-/-}$ vs. $94.4 \pm 5.9$ picomole/10$^7$ cells for controls, $p = .02$; Figure 4A) or thrombin ($67.9 \pm 11.1$ vs. $97.5 \pm 10.2$ picomoles/10$^7$ cells, $p = .04$; Figure 4B) from Ca$_{3.2}^{-/-}$ platelets was significantly reduced.

Defective ATP release could be due to less ATP being available for release or less ATP in Ca$_{3.2}^{-/-}$ platelets. Therefore, we assessed the amount of releasable ATP and total ATP content in platelets. In response to thrombin (2 U/ml), ATP released from Ca$_{3.2}^{-/-}$ platelets was significantly reduced.

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Cav3.2$^{-/-}$ platelets showed significantly reduced P-selectin exposure compared with controls ($1058 \pm 102.2$ mean fluorescence intensity (MFI) vs. $577 \pm 41.2$ MFI, $p < .001$; Figure 4C,D). ATP release induced by collagen (0.8 $\mu$g/ml) and thrombin (10 mU/ml) was significantly reduced in Cav3.2$^{-/-}$ platelets ($p = .02$, WT vs. Cav3.2$^{-/-}$; Figure 4E,F).

**FIGURE 3** ERK activation is reduced in Ca$_{3.2}^{-/-}$ and Ni$^{2+}$-treated platelets. (A) Representative western blot image showing reduced pERK level in Ca$_{3.2}^{-/-}$ platelets activated with thrombin (10 mU/ml). (B) Quantification of pERK/tERK ratio (*$p = .01$, WT vs. Ca$_{3.2}^{-/-}$). (C) Representative western blot image showing reduced pERK level in Ni$^{2+}$-treated WT platelets activated with thrombin (10 mU/ml). (D) Quantification of pERK/tERK ratio (*$p = .04$, vehicle vs. Ni$^{2+}$). (E-G) Platelet granule release and integrin $\alpha_{\text{IIb}}\beta_3$ activation mediated by thrombin (10 mU/ml). (E) ATP release (*$p = .03$, vehicle vs. Ni$^{2+}$). (F) P-selectin exposure (*$p = .001$, vehicle vs. Ni$^{2+}$) and (G) activated $\alpha_{\text{IIb}}\beta_3$ (*$p = .01$, vehicle vs. Ni$^{2+}$) in Ni$^{2+}$-treated platelets. Data are mean $\pm$ SEM and were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. ANOVA, analysis of variance.
intensity [MFI] vs. 1306.6 ± 107.3 MFI, \( p = .001 \); Figure 4C). Activated α_{IIb}β3 amplifies activation signals and platelet aggregation.\(^{39}\) Similarly, α_{IIb}β3 activation was significantly reduced in Ca_{v3.2}−/− versus control platelets (1991.5 ± 214.6 MFI vs. 2876 ± 234.8 MFI, \( p = .001 \); Figure 4D). The expression of integrin and platelet receptors was intact in Ca_{v3.2}−/− platelets (Figure S3). These findings suggest that Ca_{v3.2}−/− platelets have granule-release and α_{IIb}β3-activation defects.
Similarly, Ni²⁺ significantly decreased ATP release in Ca_{3.2}^{−/−} versus control platelets (79.7 ± 5.6 picomole/10^7 cells vs. 104.1 ± 8.6 picomole/10^7 cells, p = .03; Figure 4E) as well as P-selectin exposure (838.6 ± 62.3 vs. 1081.6 ± 69.8 MFI, p = .001; Figure 4F). Ni²⁺ also reduced the activation of integrin αIIbβ3 in Ca_{3.2}^{−/−} versus control platelets (1650.9 ± 84.9 MFI vs. 2287.6 ± 184.1 MFI, p = .01; Figure 4G). We treated Ca_{3.2}^{−/−} platelets with Ni²⁺ (30 μM) to investigate whether Ni²⁺ can induce a further reduction in the secretion of Ca_{3.2}^{−/−} platelets. As expected, Ni²⁺ had a minimal effect on ATP release from Ca_{3.2}^{−/−} platelets (Figure S4). Our results suggest that Ca_{3.2} regulates platelet granule secretion and activation.

FIGURE 5 Collagen- and thrombin-mediated aggregation is impaired in Ca_{3.2}^{−/−} platelets. Aggregation of washed platelets in the presence of calcium (2 mM) using light transmission Chrono-log aggregometer. Comparison of WT and Ca_{3.2}^{−/−} platelets mediated by (A) collagen (0.8 μg/ml, *p = .03, WT vs. Ca_{3.2}^{−/−}) and (B) thrombin (10 mU/ml, *p = .04, WT vs. Ca_{3.2}^{−/−}) and (C) collagen 1 μg/ml and (D) thrombin 20 mU/ml. Data are mean ± SEM (n = 3–5) and were analyzed by unpaired t-test with Mann-Whitney U test. ANOVA, analysis of variance; WT, wild-type.
3.5 | Collagen- and thrombin-mediated aggregation is defective in Ca$_{v3.2}^{-/-}$ platelets

Platelet secretion is important for aggregation.\textsuperscript{40} Importantly, released ADP amplifies activation signals, thus enhancing aggregation.\textsuperscript{40,41} Defective platelet granule release may affect platelet aggregation. When activated with collagen, Ca$_{v3.2}^{-/-}$ platelets showed reduced aggregation compared with wild-type controls (0.8 μg/ml; 47 ± 6% vs. 68 ± 4%, p = .03; Figure 5A) and thrombin (10 mU/ml; 24 ± 4% vs. 37 ± 4%; p = .04; Figure 5B). However, collagen 1 μg/ml (Figure 5C) or thrombin 20 mU/ml (Figure 5D), mediated similar aggregation of Ca$_{v3.2}^{-/-}$ and wild-type platelets.

NiCl$_2$ inhibits human platelet aggregation.\textsuperscript{42} Ni$^{2+}$ (30 μM) reduced mouse platelet aggregation induced by thrombin (10 mU/ml; 23 ± 2% for Ni$^{2+}$-treated platelets vs. 34 ± 4% for vehicle controls, p = .03; Figure 6A). Ni$^{2+}$ dose-dependently inhibited platelet aggregation (Figure S5A). Aggregation induced by high thrombin (20 mU/ml) was not attenuated by Ni$^{2+}$ (Figure 6B). Furthermore, Ni$^{2+}$ treatment had no effect on the aggregation of Ca$_{v3.2}^{-/-}$ platelets (Figure S5B). Thus, Ca$_{v3.2}$ may be important for platelet aggregation.

3.6 | Ca$_{v3.2}$ T-type calcium channel regulates FeCl$_3$-induced arterial thrombosis

Next, we performed FeCl$_3$-induced arterial thrombosis assay.\textsuperscript{43} Occlusion time was significantly increased in Ca$_{v3.2}^{-/-}$ (global) mice versus wild-type controls (11.93 ± 1.5 min vs. 8.88 ± 2.3 min, p = .019; Figure 7A,B). Defective arterial thrombosis could result from abnormal endothelium, platelets, or other cell types in Ca$_{v3.2}^{-/-}$ mice. Therefore, we generated platelet-specific Ca$_{v3.2}^{-/-}$ mice by crossbreeding platelet factor 4-cre (pf4$^{cre/}$) with Ca$_{v3.2}^{fl/fl}$ mice (Figure S6). Ca$_{v3.2}^{plt^{-/-}}$ mice showed significantly increased carotid artery occlusion time versus controls (15.22 ± 4.5 min vs. 10.98 ± 2.4 min, p = .013; Figure 7C,D). Histology of the carotid artery sections showed similar FeCl$_3$ treatments (Figure S7A,B). Similarly, Ca$_{v3.2}^{-/-}$ (p = .001; Figure 7E,F) and Ca$_{v3.2}^{plt^{-/-}}$ mice (p = .001; Figure 7G,H) showed significantly reduced thrombus growth on collagen-coated surfaces in a microfluidic chamber. Both in vivo and ex vivo results highlight the role of Ca$_{v3.2}$ in thrombosis. The tail bleeding time was similar between the Ca$_{v3.2}^{-/-}$ and wild-type mice and Ca$_{v3.2}^{plt^{-/-}}$ mice and controls (146.4 ± 32.94 sec for Cav3.2$^{-/-}$, n = 10 vs. sec for 159.53 ± 23.6 WT, n = 13; p = .87; Figure S8A, and 136.5 ± 26.86 sec for Cav3.2$^{plt^{-/-}}$, n = 12 vs. 166.9 ± 23.95 sec for Ca$_{v3.2}^{fl/fl}$, n = 15; p = .31; Figure S8B), which indicates normal hemostasis.

4 | DISCUSSION

In the current study, we found that Ca$_{v3.2}$ is expressed in platelets and regulates platelet [Ca$^{2+}$], content. Ca$_{v3.2}^{-/-}$ and Ni$^{2+}$-treated platelets showed reduced calcium influx independent of released
ATP/ADP. Defects in SOCE may result in decreased calcium influx. However, thapsigargin-mediated calcium entry via SOCE, primarily Orai1, was intact in Ca3.2−/− platelets. Unlike Orai145 and ligand-activated P2X136 the activation and inactivation of Ca3.2 is voltage dependent.15,46 Overlapping of activation and inactivation curves allows for calcium influx known as a “window current” through T-type calcium channels that are open at the resting membrane potential.47 Such window currents regulate calcium-sensitive processes in nonexcitable cells such as vascular endothelial cells47 and cortical cells of the adrenal cortex.18 T-type calcium channels allow calcium influx in slightly depolarized nonexcitable cells.38 Platelet membrane potential at rest is −60 mV44, suitable for the window current,18,47 and agonist-mediated changes in platelet membrane potential may allow calcium entry through Ca3.2. Studies suggest that changes in membrane potential regulate calcium entry.44,48,49 Moreover, thrombin and collagen mediate calcium entry through T-type calcium channels in pulmonary microvascular endothelial cells and smooth muscle cells.50,51 However, further studies are required to gain insights into how calcium entry through Ca3.2 occurs in platelets.

Calcium mediates granule release and integrin activation through pERK,32,37,52 which is significantly reduced in Ca3.2−/− and Ni2+-treated platelets. This finding agrees with the previously reported Ca3.2-dependent ERK activation.38 Next, we assessed the platelet activation by measuring platelet secretion and αIIbβ3 activation. Although the calcium is severely reduced in SOCE-ablated platelets, activation is normal in response to thrombin.45,53 In contrast, Ca3.2−/− and Ni2+-treated platelets showed reduced platelet activation. Unlike SOCE, Ca3.2 is associated with SNARE proteins54 and expressed near secretory vesicles.55 Moreover, Ca3.2 regulates exocytosis in rat chromaffin cells, which is sensitive to Ni2+.22,23 A small calcium surge through Ca3.2 may be sufficient to induce platelet secretion. Therefore, platelet activation defect is evident in Ca3.2−/− but not in SOCE ablated platelets.45,53 The paracrine activity of the released ATP/ADP induces activation amplification and aggregation. Therefore, we assessed platelet aggregation. As expected, Ca3.2−/− and Ni2+-treated platelets showed decreased aggregation.

Next, we performed both in vivo and ex vivo thrombosis assays. Ca3.2 deletion (global or platelet-specific) decreased arterial thrombosis and thrombus growth on a collagen-coated surface. Increased occlusion time in the absence of Ca3.2 may be due to increased embolism. Decreased thrombus growth on a collagen-coated surface indicates the possibility of embolization. However, a study of emboli formation is required to support our hypothesis. However, our tail bleeding assay indicated normal hemostasis in Ca3.2−/− mice. This discrepancy could be due to differences in injury type, site of injury, the blood vessels involved, blood flow rates, and injury-dependent activation of various pathways of the coagulation cascade.

The limitation of our study is the lack of mechanisms of Ca3.2-mediated exocytosis. However, there could be two possible mechanisms. First, the interaction of Ca3.2 with SNARE proteins governs low-threshold exocytosis, as observed in chromaffin cells.55 Both platelets and chromaffin cells share similar exocytosis mechanisms mediated by calcium influx, which could be an explanation.52 Future studies are required to unveil such association. Second, calcium influx can mediate granule exocytosis via ERK activation.32

Hypertensive and diabetic patients are at risk of cardiovascular complications and kidney disease.57–59 Aspirin has been the drug of choice to prevent cardiovascular complications58 but causes bleeding and stroke.60 Some patients are also intolerant to aspirin,38 which highlights the need for an efficient and safe antiplatelet drug. Efonidipine can improve vascular endothelial function via its role in T-type calcium channels and also has antiplatelet activity.10 Additionally, T-type calcium channel blockers have a protective effect on renal function.61,62 Our findings indicate that the development of drugs targeting Ca3.2 may help lessen the risk of cardiovascular complications.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

H.K.T., B.H.T., and C.C.C. conceived the idea for the study, designed the research, and wrote the manuscript. H.K.T. performed the experiments, collected and analyzed the data, and wrote the manuscript. S.C.H. generated the floxed mice. B.H.T. designed and performed the experiments. R.B.Y. and Y.C.T. designed the experiments. Z.H.S. performed the experiments. C.C.P performed the experiments. All authors edited and reviewed the final version of the manuscript.
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SUPPORTING INFORMATION
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