TRPM7 Kinase Controls Calcium Responses in Arterial Thrombosis and Stroke in Mice

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Objective—TRPM7 (transient receptor potential cation channel, subfamily M, member 7) is a ubiquitously expressed bifunctional protein comprising a transient receptor potential channel segment linked to a cytosolic α-type serine/threonine protein kinase domain. TRPM7 forms a constitutively active Mg^{2+} and Ca^{2+} permeable channel, which regulates diverse cellular processes in both healthy and diseased conditions, but the physiological role of TRPM7 kinase remains largely unknown.

Approach and Results—Here we show that point mutation in TRPM7 kinase domain deleting the kinase activity in mice (*Trpm7*$^{R/R}$) causes a marked signaling defect in platelets. *Trpm7*$^{R/R}$ platelets showed an impaired PIP2 (phosphatidylinositol-4,5-bisphosphate) metabolism and consequently reduced Ca^{2+} mobilization in response to stimulation of the major platelet receptors GPVI (glycoprotein VI), CLEC-2 (C-type lectin-like receptor), and PAR (protease-activated receptor). Altered phosphorylation of Syk (spleen tyrosine kinase) and phospholipase C γ2 and β3 accounted for these global platelet activation defects. In addition, direct activation of STIM1 (stromal interaction molecule 1) with thapsigargin revealed a defective store-operated Ca^{2+} entry mechanism in the mutant platelets. These defects translated into an impaired platelet aggregate formation under flow and protection of the mice from arterial thrombosis and ischemic stroke in vivo.

Conclusions—Our results identify TRPM7 kinase as a key modulator of phospholipase C signaling and store-operated Ca^{2+} entry in platelets. The protection of *Trpm7*$^{R/R}$ mice from acute ischemic disease without developing intracranial hemorrhage indicates that TRPM7 kinase might be a promising antithrombotic target.

Visual Overview—An online visual overview is available for this article. (***Arterioscler Thromb Vasc Biol*. 2018;38: 344-352. DOI: 10.1161/ATVBAHA.117.310391.)**

Key Words: blood platelets ■ store-operated calcium entry ■ stroke ■ thrombosis ■ TRPM7

TRPM7 (transient receptor potential cation channel, subfamily M, member 7) belongs to the melastatin-related transient receptor potential subfamily of transient receptor potential channels[1,2] and forms a constitutively active divalent cation-selective channel that regulates many cellular processes, including cell cycle, apoptosis, and cell death.[1,3–5] Global ablation of TRPM7 or deletion of its kinase domain results in early embryonic lethality in mice.[5,6] In vitro, TRPM7 deficiency in DT40 cells and also other cell types results in the depletion of $[Mg^{2+}]_i$ and growth arrest in the presence of normal extracellular $[Mg^{2+}]$.[1,4] TRPM7-mediated cation influx has been detected in megakaryocytes,[7] and dysfunction of TRPM7 channel, but not its kinase activity, causes macrothrombocytopenia in humans and mice because of an imbalanced Mg^{2+} homeostasis in megakaryocytes and platelets.[8]

See accompanying editorial on page 285

TRPM7 contains a cytosolic α-kinase domain with an Mg^{2+}/ATP-binding site, which is critical for the catalytic activity, and its mutation on lysine 1646 abolishes the kinase

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Nonstandard Abbreviations and Acronyms

CLEC-2	C-type lectin-like receptor
CRP	collagen-related peptide
GPCR	G-protein–coupled receptor
GPVI	glycoprotein VI
IP3	inositol triphosphate
IP3R	IP3 receptor
ITAM	immunoreceptor tyrosine-based activation motif
LAT	linker for activation of T cells
PAR	protease-activated receptor
PECAM	platelet-endothelial cell adhesion molecule
PIP2	phosphatidylinositol-4, 5-bisphosphate
PKC	protein kinase C
PKCε	protein kinase C epsilon type
PLC	phospholipase C
SOCE	store-operated Ca^{2+} entry
STIM1	stromal interaction molecule 1
Syk	spleen tyrosine kinase
tMCAO	transient middle cerebral artery occlusion
TP	thromboxane receptor
TRPM7	transient receptor potential cation channel, subfamily M, member 7
WT	wild type

activity.[4,9] Autophosphorylation of the TRPM7 kinase domain on serine 1511/1567 enhances kinase–substrate interactions leading to their serine/threonine phosphorylation.[10–13] Although it has been demonstrated that the kinase domain of TRPM7 directly binds PLC (phospholipase C) isoforms,[14] contradictory findings exist on the functional consequence of this interaction. Depending on the used heterologous expression system in vitro, GPCR (G-protein–coupled receptor)-mediated PLCβ activation can either positively or negatively modulate TRPM7 channel activity.[14–16] Recently, it has been shown that PLCγ2—an important regulator of immunoreceptor tyrosine-based activation motif (ITAM) receptor-mediated Ca^{2+} signaling—is phosphorylated by TRPM7 kinase[17]; however, the physiological significance of this modification is not fully understood. PLC-mediated PIP2 (phosphatidylinositol 4,5-bisphosphate) hydrolysis induces Ca^{2+} store depletion thereby enhancing store-operated Ca^{2+} entry (SOCE), which is regulated by functional coupling of the Ca^{2+} sensor STIM1 (stromal interaction molecule 1) and the Ca^{2+} channel Orai1 in mammalian nucleated cells and platelets.[18] Recent reports provided in vitro evidence that TRPM7 kinase regulates Ca^{2+} store content, STIM1 function, and SOCE in chicken B cells.[19]

It has been reported that mice subjected to the transient middle cerebral artery occlusion (tMCAO) model of ischemic stroke display an upregulation of TRPM7 protein expression in neurons. In contrast, downregulation of TRPM7 in the ischemic brain prevented neuronal necrosis.[20,21] Consequently, it was speculated that the increased TRPM7 channel activity may enhance Ca^{2+} influx and induce neuronal cell death in the ischemic brain.[22] However, the role of TRPM7 kinase domain in the process of thromboinflammation and stroke and the associated signaling pathways remain elusive.

Here we show that deletion of the TRPM7 kinase activity in mice (*Trpm7*$^{R/R}$) inhibits (hem)ITAM-PLCγ2 and PAR (protease-activated receptor)-PLCβ3–mediated intracellular Ca^{2+} mobilization, and partially blocks SOCE in platelets, resulting in protection of mice from arterial thrombosis and thromboinflammatory brain infarction in a model of ischemic stroke.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Mg^{2+} Homeostasis and TRPM7 Channel Activity in Kinase-Dead TRPM7 Knock-In Mice

Recently, we identified TRPM7 as a key regulator of Mg^{2+} homeostasis in megakaryocytes, which is critical for platelet biogenesis in the bone marrow (BM).[8] To dissect the role of TRPM7 kinase and channel function in vivo, a kinase-dead knock-in mouse strain was generated (*Trpm7*$^{R/R}$) carrying a point mutation (Lys^{1646} to Arg) in the Mg^{2+} ATP-binding site of the kinase domain (Figure IA in the online-only Data Supplement). In agreement with a recent report,[9] the Lys^{1646} mutation of TRPM7 kinase did not influence its channel activity in mouse embryonic fibroblast cells derived from wild-type (*WT*) and *Trpm7*$^{R/R}$ embryos (Figure IB in the online-only Data Supplement). *Trpm7*$^{R/R}$ mutant mice did not show any signs of imbalanced Mg^{2+} homeostasis because Mg^{2+} concentrations in the blood serum, bones, and platelets were unaltered (Figure IC in the online-only Data Supplement). Homozygous *Trpm7*$^{R/R}$ mice were born at a normal Mendelian distribution and developed normally. Histological analysis of different organs from *Trpm7*$^{R/R}$ mice revealed no obvious alterations (Figure ID in the online-only Data Supplement), and there was no evidence for a role of TRPM7 kinase in platelet biogenesis because platelet size, number, and morphology,[8] as well as the expression of major platelet glycoprotein receptors, including GPVI (glycoprotein VI) and CLEC-2 (C-type lectin-like receptor), and platelet life span, were indistinguishable from controls (Figure IIA and IIB in the online-only Data Supplement).

TRPM7 Kinase Regulates (hem)ITAM and PAR Receptor-Mediated Ca^{2+} Store Depletion and Is Directly Involved in SOCE

To study the functional significance of TRPM7 kinase in platelets, agonist-induced inside-out activation of integrin αIIbβ3 and P-selectin surface exposure, as a marker of α-granule release, were assessed by flow cytometry. Activation of *Trpm7*$^{R/R}$ platelets was significantly reduced in response to intermediate or low doses of thrombin and GPVI-specific agonists (collagen-related peptide [CRP], convulxin [CVX]; Figure 1A). However, *Trpm7*$^{R/R}$ platelets were able to spread on fibrinogen with similar kinetics as *WT* control platelets, indicating that TRPM7 kinase did not affect integrin outside-in signaling (Figure IIC in the online-only Data Supplement). Aggregation responses of *Trpm7*$^{R/R}$ platelets to high concentrations of either GPVI or GPCR agonists (U46619, ADP,

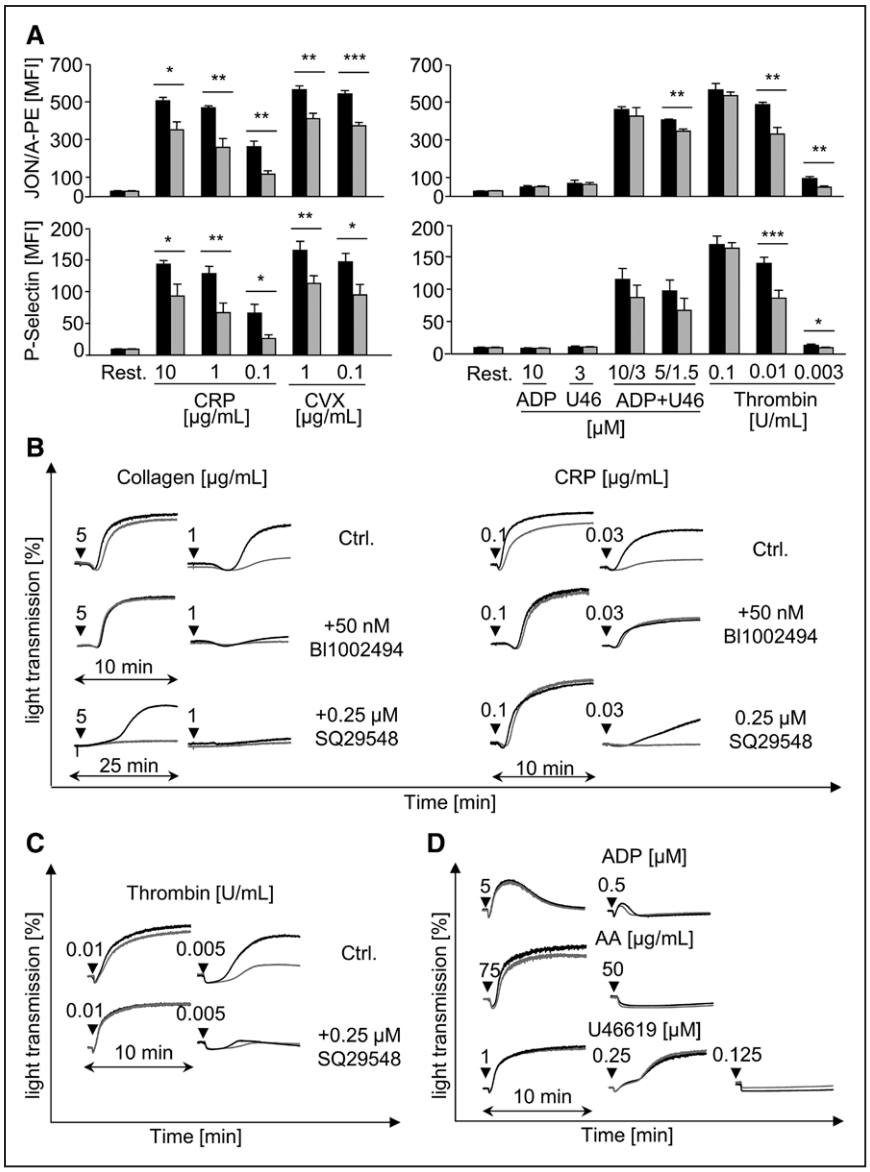

Figure 1. Defective platelet activation and aggregation responses in *Trpm7*$^{R/R}$ mice. **A**, Flow cytometric analysis of αIIbβ3 activation (**top**) and P-selectin exposure (**bottom**) in *WT* (black bars) and *Trpm7*$^{R/R}$ (grey bars) platelets. Concentrations of agonists are indicated. Results are presented as mean fluorescence intensity (MFI)±SD. **B**–**D**, Aggregation curves of *WT* (black) and *Trpm7*$^{R/R}$ (grey) platelets in response to the indicated agonists with or without the stated treatments. Washed platelets were stirred for indicated time in the presence of the indicated agonists, and light transmission was recorded with an aggregometer. ADP measurements were performed in platelet-rich plasma (PRP). U46, stable thromboxane A2 analogue U46619. BI1002494, Syk blocker; SQ29548, TP receptor blocker. Unpaired Student *t* test **P*<0.05, ***P*<0.01, ****P*<0.001. AA indicates arachidonic acid; Coll, Horm collagen; CRP, collagen-related peptide; Ctrl, control; CVX, convulxin; and Thr, thrombin.

thrombin) or arachidonic acid were normal. However, at threshold concentration of CRP, collagen, and thrombin, the aggregation response was impaired in *Trpm7*$^{R/R}$ platelets (Figure 1B). Amplification of GPVI signaling requires activation of the linker for activation of T cells (LAT) complex through spleen tyrosine kinase (Syk), as well as thromboxane A2 and ATP/serotonin release. We found that pretreatment with the Syk inhibitor BI1002494 could mimic the aggregation defects of *Trpm7*$^{R/R}$ platelets in response to GPVI agonists, whereas the TP (thromboxane receptor) blocker SQ29548 had no effect. In contrast, thrombin-dependent aggregation was normalized in the presence of SQ29548 (Figure 1B and 1C). Moreover, thrombin or CRP-mediated serotonin and ATP release from δ-granules was significantly reduced in *Trpm7*$^{R/R}$ platelets at threshold agonist concentrations, whereas thromboxane A2 release was not affected (Figure 2A; Figure IID in the online-only Data Supplement). In line with this result, pretreatment with a high dose of apyrase or supplementation of ADP normalized collagen-mediated aggregation responses of *Trpm7*$^{R/R}$ platelets (Figure IIIA in the online-only Data Supplement). Altogether, these results suggest that TRPM7 kinase is specifically involved in granule release but not in the direct activation of TP receptor or thromboxane A2 synthesis.

Activation-dependent increase in cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_i$) through the release of Ca^{2+} from intracellular stores and subsequent SOCE and receptor-operated Ca^{2+} entry are prerequisites for platelet integrin αIIbβ3 activation, as well as degranulation.[18,23] To investigate whether altered Ca^{2+} responses accounted for the impaired α- and δ-granule secretion in *Trpm7*$^{R/R}$ platelets, GPVI-ITAM and GPCR signaling were analyzed by fluorimetric determination of agonist-induced changes in $[Ca^{2+}]_i$. In line with the reduced integrin activation and degranulation, Ca^{2+}-store release was significantly reduced in *Trpm7*$^{R/R}$ platelets in response to CRP or thrombin (Figure 2B), indicating a combined GPVI and PAR receptor signaling defect on platelet activation. As a consequence of the abnormal Ca^{2+} store release, the GPVI-PLCγ2– and thrombin-PLCβ–induced Ca^{2+} influx was also reduced in *Trpm7*$^{R/R}$ platelets and could be normalized by treatment with BI1002494 (Figure 2C). To study whether

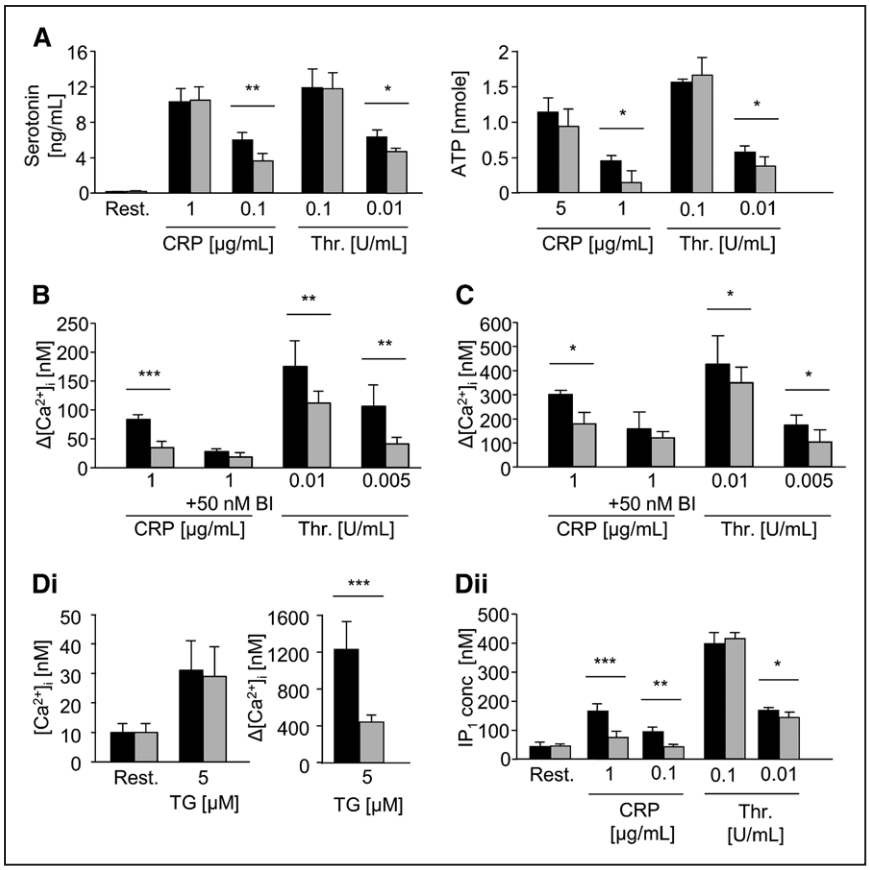

Figure 2. Reduced serotonin release, ATP release, Ca^{2+} responses, and phospholipase C activity in *Trpm7*$^{R/R}$ platelets. **A**, Measurement of platelet-released serotonin in both resting (Rest) and activated states tested by ELISA (**left**). Platelets were activated with the indicated agonists, and ATP secretion was determined (**right**). **B**, Ca^{2+} responses were measured in Fura-2-loaded platelets for store release. **C**, Ca^{2+} responses were measured in Fura-2-loaded platelets in the presence of Ca^{2+} for Ca^{2+} entry. (**Di**) Thapsigargin (TG)-induced Ca^{2+} responses were measured for store release (**left**) and store-operated Ca^{2+} entry (SOCE; **right**). Mean increase in $[Ca^{2+}]_i$ quantified by subtracting baseline levels before stimulus and finally ($\Delta[Ca^{2+}]_i$)±SD is reported. Triton-X for maximal and EDTA for minimal calcium levels were used to calibrate each measurement. (**Dii**) IP_1 (inositol monophosphate) production, as a specific metabolite of IP_3, was quantified on activation with the indicated concentrations of either collagen-related peptide (CRP) or thrombin (Thr; **right**). *WT* (black bars) and *Trpm7*$^{R/R}$ (grey bars). Unpaired Student *t* test **P*<0.05, ***P*<0.01, ****P*<0.001. BI, Syk blocker BI1002494.

TRPM7 kinase could directly contribute to the regulation of SOCE, the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin was used to bypass the GPVI- and PAR receptor-induced Ca^{2+} mobilization defect and directly modulate SOCE. Despite the normal basal cytoplasmic Ca^{2+} level and thapsigargin-induced Ca^{2+} store release, SOCE was strongly reduced in *Trpm7*$^{R/R}$ platelets, suggesting a specific role of TRPM7 kinase in the regulation of STIM1-mediated SOCE in platelets (Figure 2Di).

GPVI and PAR receptor signaling involves a series of phosphorylation events that finally enhance PLCγ2 and PLCβ activity, respectively, which mediates the hydrolysis of PIP_2 into inositol triphosphate (IP_3) and diacylglycerol.[23,24] IP_3-mediated stimulation of the IP_3R (IP_3 receptors) on intracellular Ca^{2+} store membranes leads to their activation and subsequent Ca^{2+} store depletion. Because both GPVI and PAR receptor-induced Ca^{2+} store depletion were abnormal in *Trpm7*$^{R/R}$ platelets, and the kinase domain of TRPM7 directly binds PLC isoforms,[14] IP_3 concentrations in activated platelets were determined with an indirect method using an IP_1 ELISA. In line with the other results, IP_1 production in response to CRP and low dose of thrombin were reduced in *Trpm7*$^{R/R}$ platelets, indicating a defective PIP_2 metabolism in *Trpm7*$^{R/R}$ platelets (Figure 2Dii).

The GPVI signalosome is regulated by Src family kinase Lyn and the tyrosine kinase Syk, which modulates the enzymatic activity of PLCγ2 and other signaling molecules.[25] In agreement with our previous results (Figures 1B, 2B, and 2C), we found delayed tyrosine phosphorylation of Syk$^{Y525/526}$, LAT$^{Y132/Y191}$, and PLCγ2^{Y759} in *Trpm7*$^{R/R}$ platelets upon low-dose CRP stimulation, whereas the phosphorylation was normal at high-dose CRP stimulation (Figure 3A; Figure IIIB in the online-only Data Supplement). Furthermore, phosphorylation of serine residue 729 on PKCε (PKC [protein kinase C] epsilon type)—a downstream effector of PLCγ2—was also diminished. In sharp contrast, phosphorylation of Lyn^{Y507} at the inhibitory loop of the enzyme was unaltered (Figure 3A). Based on these findings, we hypothesized that a proximal regulator, such as Syk, could be affected in Trpm7R/R platelets. In addition, PLCβ3 phosphorylation was also reduced upon stimulation with different concentrations of thrombin (Figure 3B; Figure IIIC in the online-only Data Supplement). To further address the potential role of Syk in TRPM7 kinase signaling, (hem)ITAM receptor CLEC-2 was stimulated with rhodocytin. Integrin activation, P-selectin exposure, and aggregation responses of *Trpm7*$^{R/R}$ platelets toward high concentrations of rohdocytin were normal, whereas activation responses to intermediate and lower agonist concentrations were reduced (Figure 4A and 4B). In line with this, we observed a reduced phosphorylation of Syk$^{Y525/526}$, LAT$^{Y132/Y191}$, and PLCγ2^{Y759} in *Trpm7*$^{R/R}$ platelets upon rohdocytin stimulation (Figure 4C).

TRPM7 Kinase Is Essential for Occlusive Arterial Thrombus Formation

To study whether TRPM7-mediated modulation of PLCγ2 activity plays a significant regulatory role in thrombosis and hemostasis, we next analyzed the ability of *Trpm7*$^{R/R}$ platelets to form thrombi on fibrous collagen in a heparinized whole

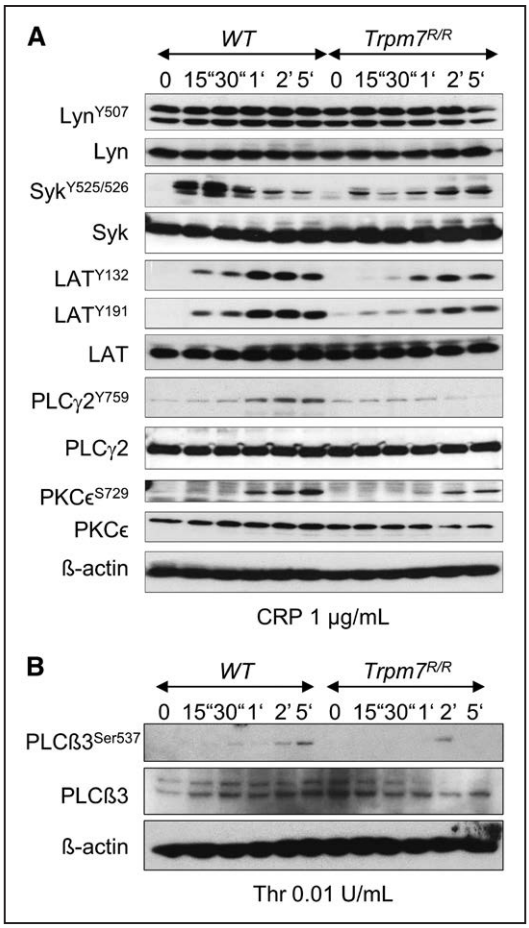

Figure 3. TRPM7 (transient receptor potential cation channel, subfamily M, member 7) kinase phosphorylates and regulates GPVI- and PAR-mediated signaling. **A**, Western blot showing the tyrosine phosphorylation pattern of *Trpm7*$^{R/R}$ platelets on collagen-related peptide (CRP) or (**B**) thrombin (Thr) stimulation. LAT indicates linker for activation of T cells; PKCε, protein kinase C epsilon type; PLCγ2, phospholipase C γ2; and Syk, spleen tyrosine kinase.

blood perfusion system to prevent thrombin generation. Here, *WT* platelets adhered to the collagen fibers and formed 3-dimensional aggregates within 2 to 4 minutes, which consistently grew resulting in large thrombi at the end of the perfusion period, whereas *Trpm7*$^{R/R}$ platelets exhibited reduced adhesion and impaired formation of 3-dimensional thrombi (Figure 5A). To test whether these defects also translated into impaired arterial thrombosis in vivo, we induced a mechanical injury on the abdominal aorta and then monitored blood flow with an ultrasonic flow probe. Although in *WT* mice, 19 of 21 (90%) arteries occluded during the observation period, only 11 of 19 (58%) *Trpm7*$^{R/R}$ arteries formed occlusive thrombi, and these showed significantly prolonged occlusion times compared with *WT* control (*WT*, 297±89 versus *Trpm7*$^{R/R}$, 454±189 s; Figure 5B). Next, thrombus formation was induced by injuring mesenteric arterioles with $FeCl_3$ and thrombus formation was monitored by intravital fluorescence microscopy. The time to first appearance of thrombi <10 µm was similar in the mesenteric arterioles of *WT* and *Trpm7*$^{R/R}$ mice (*WT*, 506±95 versus *Trpm7*$^{R/R}$, 528±117 s; Figure IV in the online-only Data Supplement). In sharp contrast, although complete vessel occlusion was observed in all arteries of *WT* mice, 15 of 26 (58%) vessels in *Trpm7*$^{R/R}$ mice did not occlude (mean time to occlusion: *WT*, 966±234 versus *Trpm7*$^{R/R}$, 1236±339 s; Figure 5C, left; Movies I and II in the online-only Data Supplement). This defect seemed to result from thrombus instability because microaggregates were frequently released from the surface of *Trpm7*$^{R/R}$ thrombi. Furthermore, *Trpm7*$^{R/R}$ mice were fully protected in a third thrombosis model, where thrombus formation was induced in the carotid arteries by topical application of $FeCl_3$ and blood flow is monitored by an ultrasonic flow probe (Figure 5C, right). Besides defective arterial thrombus formation, we also observed a prolonged tail bleeding time in *Trpm7*$^{R/R}$ mice (mean bleeding time of *WT*, 338±261 versus *Trpm7*$^{R/R}$, 638±332 s; Figure 5D), demonstrating an important role of TRPM7 kinase in primary hemostasis. These findings revealed that TRPM7 kinase-dependent modulation of GPVI signaling is a critical determinant for thrombus growth and stability in vivo.

TRPM7 Kinase Plays an Important Role in Ischemic Stroke

To assess a possible functional role of TRPM7 kinase in the pathogenesis of acute ischemic brain infarction, we next analyzed neuronal damage in *WT* and *Trpm7*$^{R/R}$ mice subjected to tMCAO. In this model of ischemic stroke, a filament is advanced to the middle cerebral artery to occlude the vessel for 1 hour followed by reperfusion after removal of the filament, which typically results in further infarct growth during the next 24 hours (reperfusion injury).[26] Strikingly, infarct volumes in *Trpm7*$^{R/R}$ brains after 24 hours were dramatically reduced to <40% of those seen in *WT* controls (Figure 6A). To determine whether this protective effect was because of an increased resistance of *Trpm7*$^{R/R}$ neurons to ischemia or to altered blood cell function, BM chimeras were generated by injecting *WT* BM cells into lethally irradiated *Trpm7*$^{R/R}$ mice and vice versa. Remarkably, both, *Trpm7*$^{R/R}$ animals substituted with *WT* BM and *WT* animals substituted with *Trpm7*$^{R/R}$ BM cells developed significantly smaller infarcts compared with *WT* animals that received *WT* BM cells (*WT/BM*^{WT}, 97±22 versus *Trpm7*$^{R/R}$*/BM*^{WT}, 47±11 versus *WT/BM*$^{Trpm7R/R}$, 59±35 mm^3; ***P=0.001; Figure 6B, left). Importantly, in serial magnetic resonance imaging on day 7 after tMCAO, we did not observe delayed infarct progression or intracranial hemorrhage (Figure 6B, right). The reduction in infarct size was functionally relevant because global neurological (Bederson score) and motor (Grip test) functions (Figure 6C) were improved in *Trpm7*$^{R/R}$*/BM*^{WT} and *WT/BM*$^{Trpm7R/R}$ mice, when compared with *WT* mice 1 day after tMCAO. Infarct growth in the acutely ischemic brain involves closely intertwined inflammatory and thrombotic pathomechanisms, referred to as thromboinflammation, in which platelets are key mediators.[27] To further clarify the extent to which altered platelet function contributed to the protection of *WT/BM*$^{Trpm7R/R}$ mice from tMCAO-induced neuronal damage, *WT* mice rendered thrombocytopenic by anti-GPIbα (glycoprotein Ib alpha) antibody injection were transfused with *WT* or *Trpm7*$^{R/R}$ platelets and subsequently subjected to tMCAO.[28,29] Remarkably, *WT* mice that had received *Trpm7*$^{R/R}$ platelets developed significantly smaller brain infarcts compared with controls, and also global neurological and motor function were significantly improved (Figure 6D),

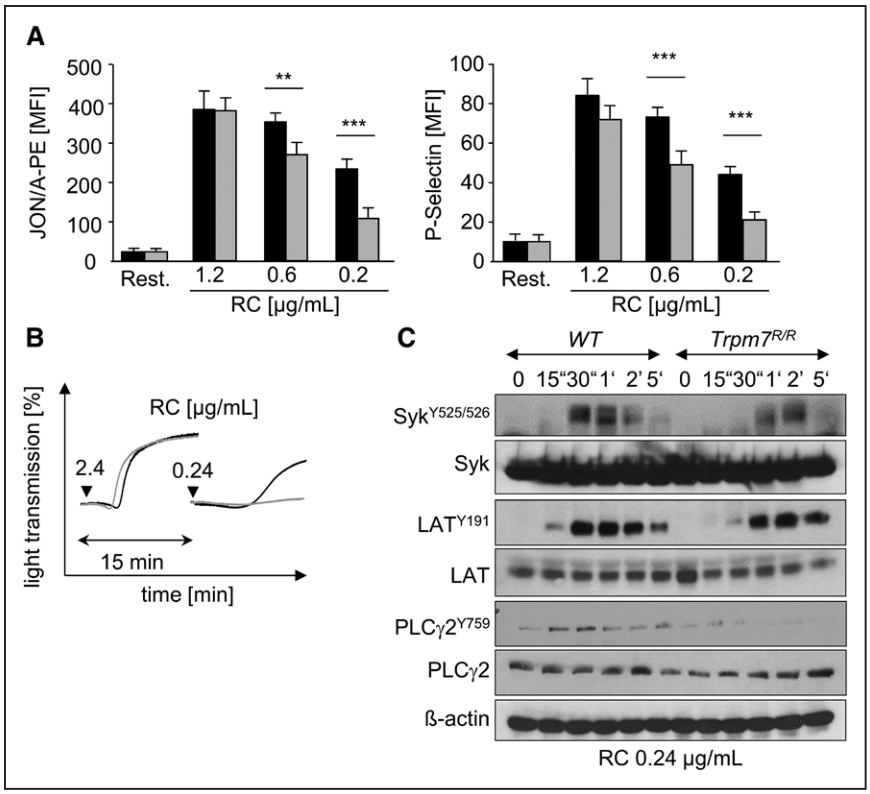

Figure 4. TRPM7 (transient receptor potential cation channel, subfamily M, member 7) kinase phosphorylates and regulates CLEC-2 signaling in platelets. **A**, Flow cytometric analysis of αIIbβ3 activation (**top**) and P-selectin exposure (**bottom**) in *WT* (black bars) and *Trpm7*$^{R/R}$ (grey bars) platelets on rhodocytin (RC) stimulation. Results are presented as mean fluorescence intensity (MFI)±SD. **B**, Aggregation curves of *WT* (black) and *Trpm7*$^{R/R}$ (grey) platelets in response to RC stimulation presented. **C**, Western blot showing the tyrosine phosphorylation pattern of *Trpm7*$^{R/R}$ platelets on RC stimulation. Unpaired Student *t* test $^{**}P<0.01$, $^{***}P<0.001$. LAT indicates linker for activation of T cells; PLCγ2, phospholipase C γ2; Rest, resting; and Syk, spleen tyrosine kinase.

clearly demonstrating that TRPM7 kinase activity in platelets contributes to the development of ischemic brain infarction.

Discussion

The α-kinase protein family comprises various members including TRPM6,[30] TRPM7,[31] and eEF2 serine/threonine kinases.[32] To date, only few substrates of TRPM7 kinase have been identified in vitro, namely myosin IIa,[33] PLCγ2,[17] and annexin-1.[12] Although tyrosine kinase-dependent platelet signaling has been extensively studied, the physiological importance of serine/threonine phosphorylation in ITAM receptor signaling triggered by TPRM7 kinase or other α-kinases is entirely unknown. The GPVI signalosome is regulated by Src family kinases, which bind the cytoplasmic tail of GPVI,[34]

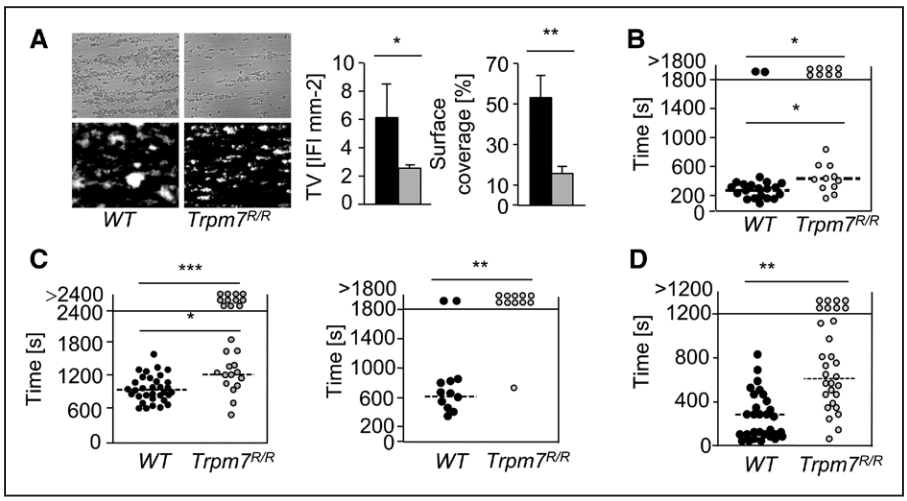

Figure 5. Impaired thrombus formation in *Trpm7*$^{R/R}$ mice. **A**, Heparinized whole blood from *WT* (black bars) and *Trpm7*$^{R/R}$ (grey bars) mice was perfused over immobilized collagen at a shear rate of 1000 s^{-1}. **Left**, representative phase-contrast images and fluorescent images (anti-GPIX-DyLight-488; **bottom**) are shown. **Right**, relative thrombus volume (TV) and mean surface coverage±SD (**left**) of *WT* (black) and *Trpm7*$^{R/R}$ (grey) are shown. **B**, The abdominal aorta in *WT* and *Trpm7*$^{R/R}$ mice was injured by tight compression with forceps, and blood flow was monitored for 30 min with an ultrasonic flow probe. The time to stable vessel occlusion is shown. **C**, Thrombus formation in $FeCl_3$-injured mesenteric arterioles was monitored by intravital microscopy of fluorescently labeled platelets. Time to stable vessel occlusion is shown (**left**), and thrombus formation in $FeCl_3$-injured carotid arteries (**right**) is presented. **D**, Tail bleeding times shown in *WT* and *Trpm7*$^{R/R}$ mice. Each symbol represents 1 individual or 1 mesenteric arteriole. Fisher exact test for assessment of differences between open and occluded vessels and Student *t* test for the comparison of mean occlusion times were used. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. IFI indicates integrated fluorescence intensity.

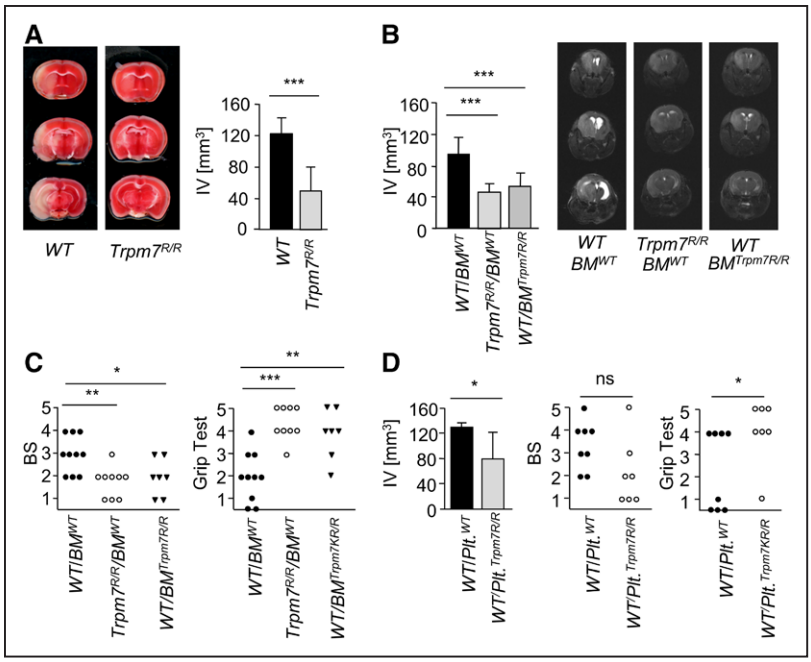

Figure 6. *Trpm7*$^{R/R}$ mice are protected from cerebral ischemia. **A**, Representative images of 3 corresponding coronal sections of *WT* and *Trpm7*$^{R/R}$ mice stained with 2,3,5-triphenyltetrazolium chloride (TTC) 24 h after tMCAO (**left**). Brain infarct volume (IV) in *WT* and *Trpm7*$^{R/R}$ mice is shown (**right**). **B**, IV of bone marrow (BM) chimeric mice. *WT* mice transplanted with either *WT* BM (*WT*/*BM*^{WT}) or *Trpm7*$^{R/R}$ mice transplanted with *WT* BM (*Trpm7*$^{R/R}$/*BM*^{WT}) or *WT* with *Trpm7*$^{R/R}$ BM (*WT*/*BM*$^{Trpm7R/R}$; **left**). Intracranial hemorrhage was assessed 7 d post-tMCAO using serial magnetic resonance imaging in the ischemic area (**right**). **C**, Neurological outcome studied by Bederson score (BS) and Grip test in *WT* and *Trpm7*$^{R/R}$ BM chimeric mice 24 h post-tMACO. **D**, Brain IVs and functional outcome (**left**) 24 h after tMCAO in platelet-depleted *WT* mice, reconstituted with either *WT* or *Trpm7*$^{R/R}$ platelets (*WT*/*Plt*^{WT}: platelet-depleted *WT* mice transfused with *WT* platelets; *WT*/*Plt*$^{Trpm7R/R}$: platelet-depleted *WT* mice transfused with *Trpm7*$^{R/R}$ platelets). Each symbol represents 1 animal. Unpaired Student *t* test or Mann–Whitney *U* test (BS and grip test) **P*<0.05, ***P*<0.01, ****P*<0.001.

and are critical for initial activation of the GPVI signalosome. However, they can also inhibit platelet activation through phosphorylation of immunoreceptor tyrosine-based inhibition motifs on PECAM (platelet-endothelial cell adhesion molecule)-1 and modulation of PKC activity.[35,36] The tyrosine kinase Syk transduces the signal from the GPVI/FcRγ (Fc receptor gamma)-chain to LAT, which in turn induces the recruitment of different adaptors and PLCγ2 from the cytosol. It has been shown that several PLC isoforms can directly bind the kinase domain of TRPM7, and that PLCγ2 is phosphorylated by TRPM7 kinase, but the physiological in vivo relevance of these interactions remained elusive.[2,14,17] Our study now demonstrates that ablation of TRPM7 kinase activity strongly inhibited the tyrosine phosphorylation cascade downstream of GPVI and CLEC-2 involving the Syk-LAT- PLCγ2 signaling axis. Because pretreatment of *Trpm7*$^{R/R}$ platelets with Syk inhibitor BI1002494 normalized aggregation responses, we speculate that TPRM7 kinase indeed regulates Syk or upstream effectors in the (hem) ITAM signaling complex. In addition, PLCβ3 activity is regulated by TRPM7 kinase, which suggests an essential role for PLC-mediated Ca^{2+} store depletion during platelet activation.

It has been shown that several phosphorylation events on serine residues of STIM1 are involved in the modulation of SOCE[37]; however, this mechanism and the regulatory kinases have not been investigated in platelets. Recently, using heterologous expression in DT40 cells, it has been shown that TRPM7 regulates SOCE. Furthermore, Ca^{2+} store content and the refilling process of the Ca^{2+} store are also regulated by TRPM7, indicating a functional link between TRPM7 and STIM1. In line with this, we show in vivo that TRPM7 kinase activity indeed regulates SOCE in platelets. However, further investigation is required to establish the exact molecular mechanisms by which the channel and kinase domain of TRPM7 regulate STIM1-mediated SOCE.

TRPM7 has been shown to be expressed in the human vascular system and to modulate Mg^{2+} homeostasis, cell growth, and proliferation in vascular smooth muscle and endothelial cells[38] and abnormal expression of the channel has been linked to the development of cardiovascular diseases.[39] In the present study, we demonstrate that the kinase activity of TRPM7 regulates platelet activation in primary hemostasis and arterial thrombosis, establishing the TRPM7 kinase as a potential target to modulate platelet activity in thromboinflammatory disease settings. Our results suggest that the combined GPVI signaling defects and reduced SOCE account for the thrombus instability and antithrombotic protection of *Trpm7*$^{R/R}$ mice.

Moreover, a balanced Ca^{2+} and Mg^{2+} homeostasis plays a pivotal role in neuronal survival under ischemic conditions. Indeed, Mg^{2+} was found to be a potent Ca^{2+} antagonist thereby protecting ischemic neurons from Ca^{2+} overload.[40] In support of this, a reduced intracellular Mg^{2+} concentration was detected in the infarcted rat and human brain, and conversely experimental hypomagnesaemia resulted in increased infarct volumes after photothrombosis.[41–43] Using BM chimeras and adoptive platelet transfer, we could decipher the contribution of TRPM7 kinase function in platelets from its function in endothelial cells and neurons. The improved motor function and reduced infarct volumes in *Trpm7*$^{R/R}$/*BM*^{WT} mice clearly showed that besides platelets, TRPM7 kinase activity in brain tissue, most likely in neurons and glia cells, may also be a critical factor for the progression of ischemic brain infarction.[20] Interestingly, in *WT* mice subjected to tMCAO, a 2-to-3–fold increase in TRPM7 expression has been observed in the cortex after reperfusion,[44,45] which likely resulted in a Ca^{2+} overload and contributed to neuronal death, as well as infarct progression.[46] In support of this, knockdown of TRPM7 prevented neuronal cell death under ischemic conditions in vivo.[21] Based on these tMCAO studies, inhibition of TRPM7 channel activity was proposed as a promising potential therapeutic target.[47] Surprisingly, our results showed that disruption of TRPM7 kinase activity either in platelets or in neurons is sufficient to protect the brain from the thromboinflammatory cerebral

I/R injury. These results suggest that under pathological conditions, increased TRPM7 channel activity cannot overcome the protective effect of abolished TRPM7 kinase function in *Trpm7$^{R/R}$* mice. Consequently, we conclude that TRPM7 kinase activity, which further accelerates PLC-mediated Ca^{2+} mobilization and SOCE, rather than enhanced TRPM7 channel activity, plays a dominant role in the development of stroke. The finding that selective disruption of TRPM7 kinase function does not cause intracranial bleeding in the ischemic brain and only moderately influences hemostasis in mice highlights TRPM7 kinase as a potential therapeutic target to develop safe antithrombotic or anti-thromboinflammatory agents.

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Disclosures

None.

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Highlights

- TRPM7 (transient receptor potential cation channel, subfamily M, member 7) kinase regulates GPVI (glycoprotein VI), CLEC-2 (C-type lectin-like receptor), and PAR (protease-activated receptor)-mediated signaling through phospholipase C activation and store-operated Ca^{2+} entry.
- Because of the impaired platelet reactivity, Trpm7R/R mice were protected in different models of arterial thrombosis and in the transient middle cerebral artery occlusion model of ischemic stroke.