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Ion channels in the regulation of platelet migration

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ABSTRACT

Platelets have been shown to migrate and thus to invade the vascular wall. Platelet migration is stimulated by SDF-1. In other cell types, migration is dependent on Ca^{2+} entry via Ca^{2+} channels. Ca^{2+} influx is sensitive to cell membrane potential which is maintained by K^+ channel activity and/or Cl^- channel activity. The present study explored the role of ion channels in the regulation of SDF-1 induced migration. Platelets were isolated from human volunteers as well as from gene targeted mice lacking the Ca^{2+} activated K^+ channel SK4 ($sk4^{-/-}$) and their wild type littermates ($sk4^{+/+}$). According to confocal microscopy human platelets expressed the Ca^{2+} channel Orai1 and the Ca^{2+}-activated K^+ channel $K_{Ca}3.1$ (SK4). SDF-1 (100 ng/ml) stimulated migration in human platelets, an effect blunted by Orai1 inhibitors 2-aminoethoxydiphenyl borate 2-APB (10 μM) and SKF-96365 (10 μM), by unspecific K^+ channel inhibitor TEA (30 mM), by SK4 specific K^+ channel blocker clotrimazole (10 μM), but not by Cl^- channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid NPPB (100 μM). Significant stimulation of migration by SDF-1 was further observed in $sk4^{+/+}$ platelets but was virtually absent in $sk4^{-/-}$ platelets. In conclusion, platelet migration requires activity of the Ca^{2+} channel Orai1 and of the Ca^{2+} activated K^+ channel SK4, but not of NPPB-sensitive Cl^- channels.

1. Introduction

Blood platelets have the capacity to migrate into the vascular wall [1–6]. The migration of platelets is stimulated by stromal cell derived factor-1 (SDF-1) [6], an inflammatory chemokine stored in platelet granules [7–9] and highly expressed in atherosclerotic plaques [10]. SDF-1 activates the CXCR4-receptor [11] and induces chemotactic migration of several cell types [12–15].

In other cell types, e.g. mast cells or neutrophils, migration is dependent on an increase of intracellular Ca^{2+} activity, which results from activation of Orai1, the pore forming unit of the store operated Ca^{2+} entry (SOCE) [16–21]. Orai1 is expressed in platelets and is critically important for platelet activation [22,23]. Its role in platelet migration has hitherto not been elucidated.

The driving force for Ca^{2+} entry is in part determined by the cell membrane potential, which is established and maintained by K^+ efflux through K^+ channels. K^+ channels serving this function in other cell types include the intermediate Ca^{2+} activated K^+ channel K(Ca)3.1 (SK4), which is expressed in several tissues [24–28]. Expression of SK4 in platelets has, however, hitherto not been explored. Cell membrane potential could further be maintained by Cl^- channels [29–32].

The present study explored the impact of ion channels on SDF-1 induced platelet migration. To this end platelet migration towards the inflammatory chemokine SDF-1 was determined in the presence and absence of the Orai1 inhibitors 2-aminoethoxydiphenyl borate (2-APB) [33–35] and SKF-96365 [36,37], the unspecific K^+ channel inhibitor tetraethylammonium (TEA) [38], the $K_{Ca}3.1$/SK4 specific K^+ channel inhibitor clotrimazole [39], as well as the unspecific Cl^- channel inhibitor 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) [40–43]. The specific role of $K_{Ca}3.1$/SK4 in platelet migration was further analysed in murine platelets isolated from gene-targeted mice lacking functional SK4 ($sk4^{-/-}$) and the corresponding wild type littermate mice ($sk4^{+/+}$).

2. Material and methods

2.1. Preparation of human platelets

Blood was drawn from volunteers or obtained from the blood bank of the university hospital. All procedures were approved by the Ethical Commission of the University. Washed platelets were isolated from acid citrate dextrose (ACD)-anticoagulated human blood as described previously [44,45]. Analysis of platelet purity

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by a whole blood analyzer (Sysmex) showed no detection of cells other than platelets. Prior to the platelet experiments, a sample of the isolated cell population was measured by flow cytometry for the platelet-specific markers CD42b and P-Selectin to assure platelet purity and to rule out preactivation. Platelets were stored in IMDM medium or phosphate-buffered saline until further use within 30 min.

2.2. Mice

Gene targeted mice lacking functional SK4 ($sk4^{-/-}$) and the corresponding wild type littermate mice ($sk4^{+/+}$) were generated and bred as described earlier [46]. Animals were genotyped by polymerase chain reaction (PCR). All animal experiments were conducted according to German law for the welfare of animals and were approved by local authorities (PY 1/10).

2.3. Preparation of murine platelets

Platelets were obtained from 10- to 12-week-old $sk4^{-/-}$ mice and $sk4^{+/+}$ mice of either sex. The mice were anesthetized with ether and blood was drawn from the retro-orbital plexus into heparinised tubes [22,47]. Blood parameters were analyzed with pocH-100iv automatic hematology analyzer (Sysmex). Platelet rich plasma (PRP) was obtained by centrifugation at 260*g* for 5 min. Afterwards PRP was centrifuged at 640*g* for 5 min to pellet the platelets. To ease platelets apyrase (0.02 U/ml, Sigma–Aldrich) and prostaglandin I_2 (0.5 μM, Calbiochem) were added to the

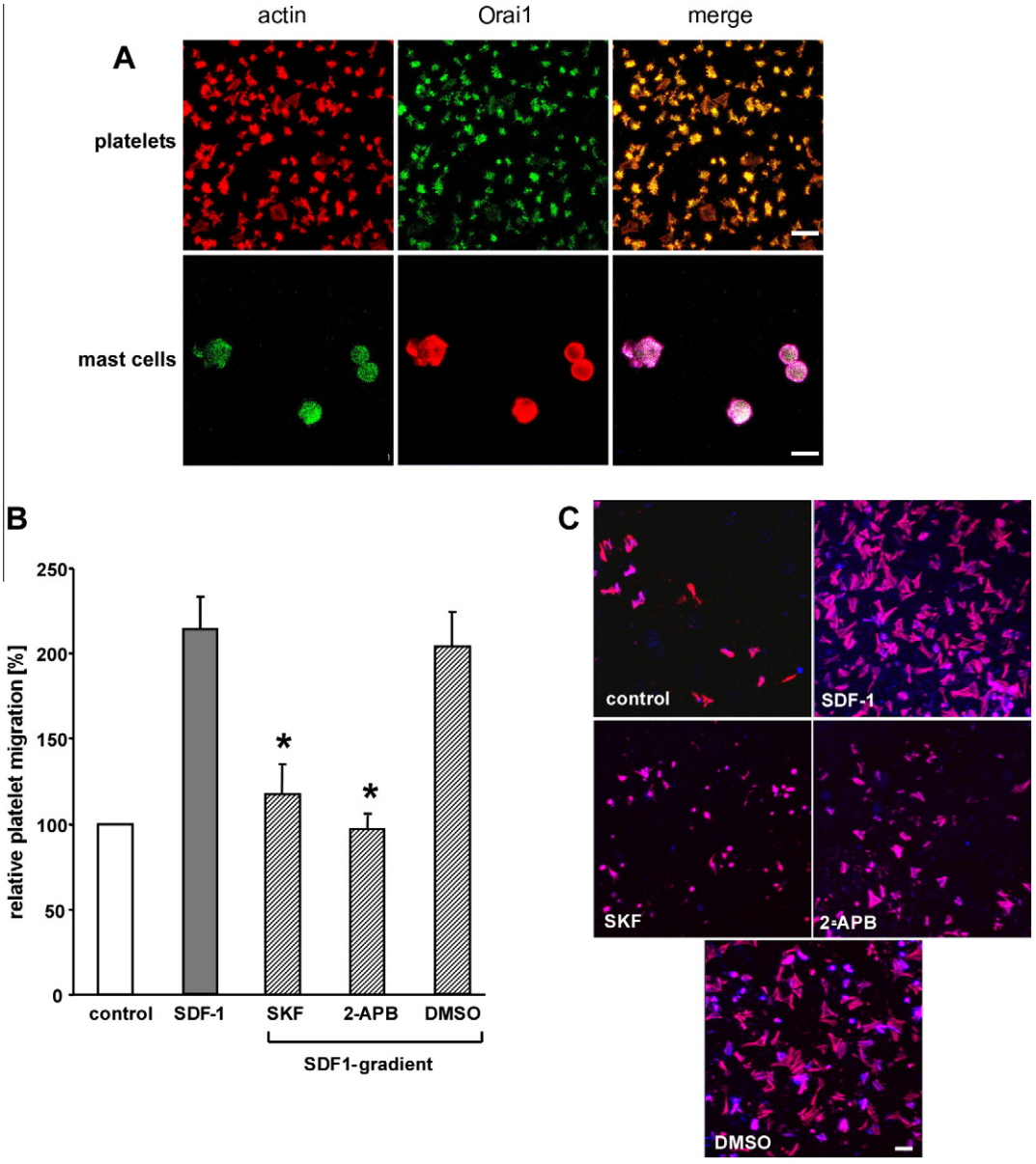

Fig. 1. Effect of SDF-1 on migration of human platelets in the absence and presence of Ca^{2+} channel blockers. (A) Confocal microscopy of Orai1 abundance in human platelets and murine mast cells as a positive control. Green: Orai1, red: actin. Scale bar equals 10 μm. (B) Arithmetic means ± SEM of migrated platelets (in percent of migration in the absence of stimulators and inhibitors, $n = 4$) following exposure to SDF-1 (100 ng/ml) in the absence of inhibitors (grey bar) and in the presence of the Orai1-channel inhibitors SKF-96365 (10 μM) or 2-APB (10 μM) and DMSO as solvent control. *($p < 0.05$) indicates statistically significant difference to value in the absence of inhibitors. (C) Representative original photographs of migrated platelets following exposure to SDF-1 (100 ng/ml) in the absence of inhibitors, in the presence of the Orai1-channel inhibitors SKF-96365 (10 μM) or 2-APB (10 μM) or in the presence of DMSO as solvent control. Scale bar equals 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PRP. After two washing steps the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM $CaCl_2$).

2.4. Transwell experiments

For transmigration experiments, we used transwell inserts (24-well, Falcon) separating the upper and the lower chamber with a membrane with pores that were 0.4 μm in diameter. The lower chamber contained SDF-1 (100 ng/ml, R&D Systems) or solvent control in IMDM medium. Murine (5×10^6 plts/ml) or human platelets (2×10^6 plts/ml) were carefully transferred into the upper chamber and were allowed to migrate through the membrane for 7 h in IMDM medium under cell culture conditions [48]. Then the platelet suspension in the lower compartment was stained with an antibody against glycoprotein Ibα (GPIbα, platelet-specific; Beckman Coulter, Immunotech). Platelets were counted by flow cytometry (BD FACS Calibur) in the platelet gate. Flow cytometric readout was confirmed by visual counting of platelets with a hemocytometer. Platelets adherent to the bottom side of the membrane were counted under the microscope and included in the analysis. Relative transmigration was calculated against control medium in the lower compartment, representing random and undirected platelet transmigration (set as 100%). The vitality of inhibitor-treated platelets was assured by trypan blue exclusion.

2.5. Culture of bone marrow-derived mast cells (BMMCs)

Femoral BMMCs from 6- to 12-week-old wild type mice were cultured for 4 weeks in RPMI 1640 medium (Gibco) containing

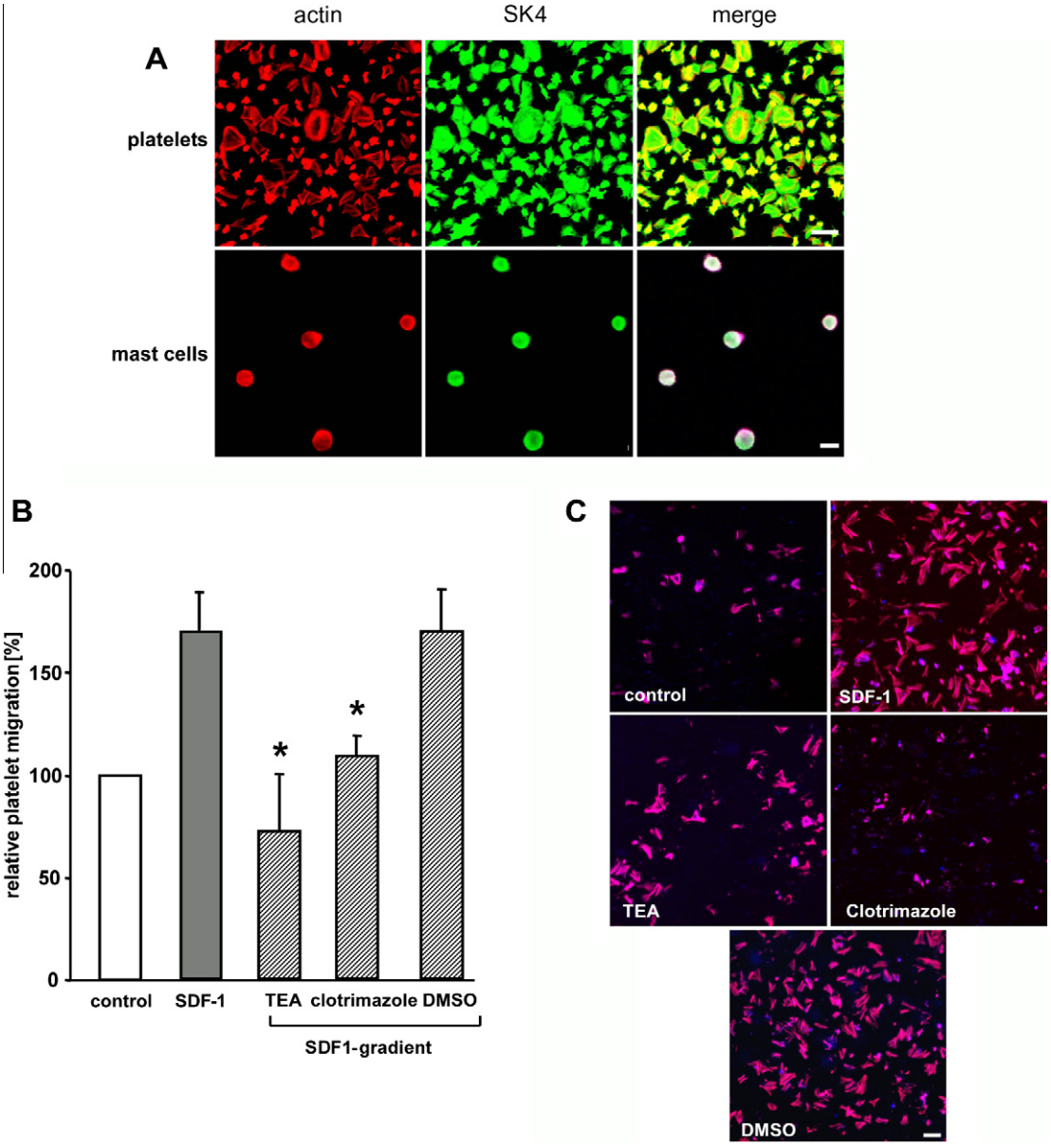

Fig. 2. Effect of SDF-1 on migration of human platelets in the absence and presence of K^+ channel blockers. (A) Confocal microscopy of the SK4 protein abundance in human platelets and murine mast cells as a positive control. Green: SK4, red: actin. Scale bar represents 10 μm. (B) Arithmetic means ± SEM of migrated platelets (in percent of migration in the absence of stimulators and inhibitors, $n = 4$) following exposure to SDF-1 (100 ng/ml) in the absence of inhibitors (grey bar), in the presence of the K^+ channel inhibitors TEA (30 mM) or clotrimazole (10 μM) and with DMSO as solvent control. *($p < 0.05$) indicates statistically significant difference to value in the absence of inhibitors. (C) Representative original photographs of migrated platelets following exposure to SDF-1 (100 ng/ml) in the absence of inhibitors and in the presence of the K^+ channel inhibitors TEA (30 mM) or clotrimazole (10 μM). Scale bar equals 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

10% fetal calf serum, 1% penicillin/streptomycin, 20 ng/ml interleukin-3 (R&D Systems, Hessen, Germany), and 100 ng/ml of the c-kit ligand stem cell factor (SCF; Peprotech, Rocky Hill, NJ, USA). BMMC maturation was confirmed by flow cytometry (FACS Calibur, Becton Dickinson).

2.6. Immunofluorescence and confocal microscopy

Washed platelets were allowed to adhere to a fibrinogen surface (20 μg/ml) on a chamber slide. BMMCs (4×10^4) were adhered on a cover slip by spinning. Cells were fixed with paraformaldehyde (2%), washed and blocked with 2% bovine serum albumin for 30 min, followed by incubation with the primary antibody for 2 h at room temperature. Primary antibodies against Orai1 (1:250, Abcam) or SK4 (1:250, Abcam) were used. Chamber slides were washed and incubated with an anti-rabbit conjugated FITC secondary antibody (Santa Cruz). The actin cytoskeleton was stained with rhodamine–phalloidin (Invitrogen). The slides were mounted with ProLong Gold antifade reagent (Invitrogen). Confocal microscopy was performed using a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss Micro Imaging) with a A-Plan 63× ocular.

2.7. Statistical analysis

Data are given as arithmetic means ± SEM. Statistical analysis was made utilizing Student's *t*-test for unpaired data or ANOVA as appropriate. A *p*-value less than 0.05 was considered as significant.

3. Results

The present study explored the participation of ion channels in the regulation of platelet migration. Specifically the study focused on the participation of the Ca^{2+} release activated Ca^{2+} channel Orai1 and the Ca^{2+}-sensitive intermediate conductance K^+ channel $K_{Ca}3.1$, also known as SK4.

In a first series of experiments the influence of pharmacological inhibition of Orai1 on migration was analyzed. As illustrated in Fig. 1A, Orai1 is expressed in human platelets. For comparison, the expression is shown in murine mast cells. SDF-1 (100 ng/ml) stimulated migration in human platelets, an effect, which was significantly blunted in the presence of the Orai1 inhibitors 2-APB (10 μM) and SKF-96365 (10 μM) (Fig. 1A and B). Thus, migration is dependent on Ca^{2+} entry through Orai1.

In a second series of experiments the role of K^+ channels in platelet migration was defined. As shown in immunofluorescence stainings the intermediate conductance Ca^{2+} activated K^+ channel $K_{Ca}3.1$ (SK4) is strongly expressed in human platelets. Again, for comparison, the expression of SK4 is shown in murine mast cells (Fig. 2A). Pharmacological inhibition of the channel by the SK4 inhibitor clotrimazole (10 μM) significantly decreased platelet migration. A similar inhibition was observed following administration of the less selective K^+ channel blocker TEA (30 mM) (Fig. 2A and B).

Further experiments were performed to elucidate whether migration of platelets required the activity of Cl^- channels. To this end the Cl^- channels were inhibited by the broad Cl^- channel blocker NPPB. As shown in Fig. 3, in contrast to the K^+ channel blockers, inhibition of Cl^- channels did not significantly modify migration. Thus, NPPB sensitive Cl^- channels are apparently not required for platelet migration.

To further define the role of the Ca^{2+} sensitive K^+ channel SK4 in platelet migration, experiments were performed in platelets isolated from gene targeted mice lacking functional SK4 ($sk4^{-/-}$) and from wild type littermates ($sk4^{+/+}$). As shown in Fig. 4, SDF-1 significantly stimulated migration in $sk4^{+/+}$ platelets, an effect virtually absent in $sk4^{-/-}$ platelets.

4. Discussion

The present study reveals that SDF-1-stimulated migration of platelets is sensitive to Ca^{2+} channel inhibitors 2-APB and SKF-96365 as well as K^+ channel inhibitors clotrimazole and TEA but not to Cl^- channel inhibitor NPPB.

Ca^{2+} entry into platelets is accomplished by the Ca^{2+} channel Orai1 [23], which has previously been shown to be required for platelet activation [22,23]. Recently, it could be shown that Orai1 is critically involved in migratory processes of breast tumor cells [49], vascular smooth muscle cells [50,51] or mast cells [17], but its role in platelet migration has not been elucidated so far. We could now identify Orai1 as the store operated Ca^{2+} entry (SOCE)

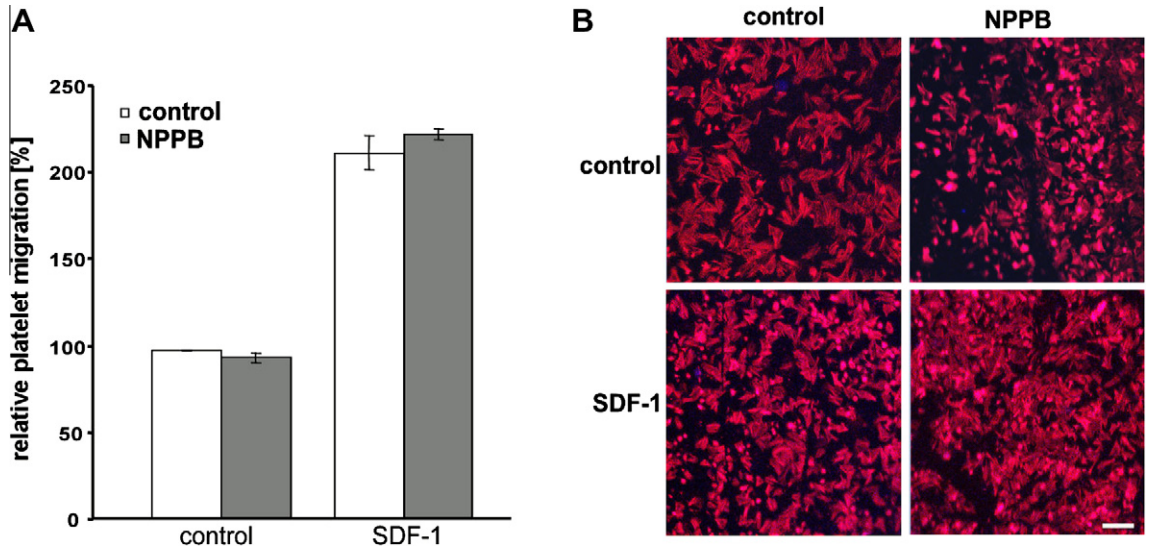

Fig. 3. Effect of SDF-1 on migration of human platelets in the absence and presence of Cl^- channel blocker NPPB. (A) Arithmetic means ± SEM of migrated platelets (in percent of migration in the absence of stimulators and inhibitors, $n = 3$) following exposure to SDF-1 (100 ng/ml) in the absence (white bar) or presence (grey bar) of 100 μM NPPB. (B) Representative original photographs of migrated platelets following exposure to SDF-1 (100 ng/ml) in the absence or the presence of NPPB (100 μM). Scale bar equals 10 μm.

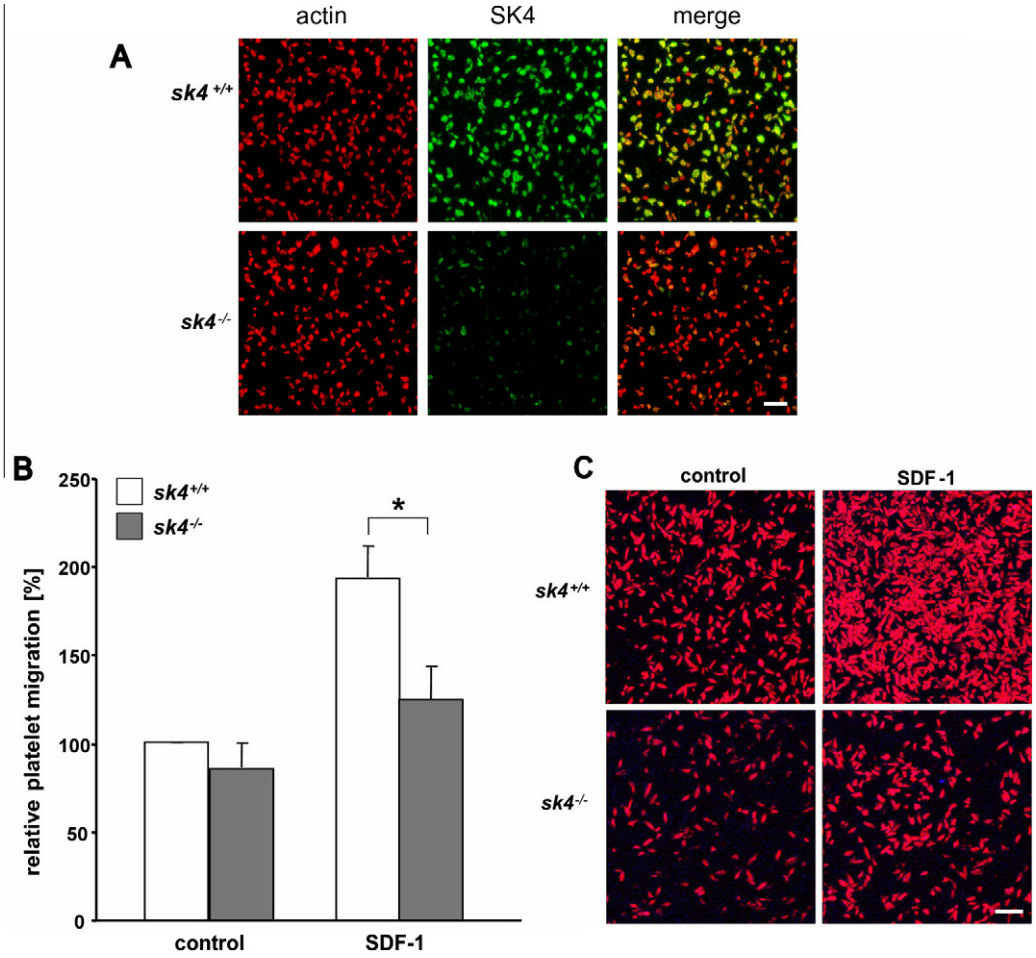

Fig. 4. Effect of SDF-1 on migration of $sk4^{+/+}$ and $sk4^{-/-}$ platelets. (A) Confocal microscopy of SK4 abundance in $sk4^{+/+}$ and $sk4^{-/-}$ platelets. Green: SK4, red: actin. Scale bar represents 10 μm. (B) Arithmetic means ± SEM of migrated platelets (in percent of spontaneous $sk4^{+/+}$ platelet migration in the absence of SDF-1, $n = 3$) from $sk4^{+/+}$ mice (white bar) and $sk4^{-/-}$ mice (grey bar) following exposure to SDF-1 (100 ng/ml). *($p < 0.05$) indicates statistically significant difference to $sk4^{+/+}$ platelets. (C) Representative original photographs of migrated platelets isolated from gene targeted mice lacking SK4 ($sk4^{-/-}$) and their wild type littermates ($sk4^{+/+}$). Scale bar equals 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

channel important for platelet migration. Accordingly, platelet migration towards SDF-1 was significantly reduced by pharmacological inhibition of SOCE.

Ca^{2+} entry through Orai1 is driven by a cell negative potential difference across the cell membrane and is thus expected to be sensitive to cell membrane potential, which could in turn be modified by K^+ channels and Cl^- channels. The present observation suggests that K^+ channels rather than Cl^- channels participate in the maintenance of the driving force. The ion channels involved are the Ca^{2+} sensitive K^+ channels, which are particularly sensitive to clotrimazole [39]. Along those lines pharmacological and genetic knockout of SK4 abrogates the stimulation of platelet migration towards the inflammatory chemokine SDF-1. Thus, regulation of migration depends on the function of both, the Ca^{2+} channel Orai1 and the Ca^{2+} sensitive K^+ channel SK4.

In an earlier study, we have shown that the stimulating effect of SDF-1 on migration involves activation of CXCR4 receptor, pertussis toxin-sensitive G-proteins, and phosphoinositide-3-kinase (PI3K) [6]. Platelet migration can further be stimulated by fMLP [1,2] and IgE [4]. It remains to be shown, whether or not those regulators of platelet migration are effective by influencing channel activity.

The inflammatory chemokine SDF-1 is strongly expressed in atherosclerotic plaques [10]. Since platelets have been shown to act as inflammatory firebugs in cardiovascular diseases [52], SDF-1-induced stimulation of platelet migration may contribute to the pathophysiology of vascular inflammation and atherogenesis. Along those lines, intestinal artery ligation and subsequent post-ischemic inflammatory reaction is followed by transmigration of platelets into the vessel wall [6]. Platelets may release inflammatory cytokines such as IL-1β [53] or SDF-1 [9] and serve as sentinel cells attracting further platelets or inflammatory cells like monocytes or stem cells [9,54]. Ion channels required for platelet migration may thus be attractive targets for the treatment of vascular inflammation or atherogenesis.

In conclusion, functional Ca^{2+} release activated Ca^{2+} channels and Ca^{2+} sensitive K^+ channels, namely $K_{Ca}3.1$/SK4, are required for the stimulation of Ca^{2+} entry into platelets which occurs following platelet stimulation with SDF-1. Inhibition of Ca^{2+} release activated Ca^{2+} channels and/or Ca^{2+} sensitive K^+ channels thus inhibits migration of platelets.

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