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RAPID REPORT

Kv1.3 is the exclusive voltage-gated K^+ channel of platelets and megakaryocytes: roles in membrane potential, Ca^{2+} signalling and platelet count

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A delayed rectifier voltage-gated K^+ channel (Kv) represents the largest ionic conductance of platelets and megakaryocytes, but is undefined at the molecular level. Quantitative RT-PCR of all known Kv α and ancillary subunits showed that only Kv1.3 (*KCNA3*) is substantially expressed in human platelets. Furthermore, megakaryocytes from Kv1.3$^{-/-}$ mice or from wild-type mice exposed to the Kv1.3 blocker margatoxin completely lacked Kv currents and displayed substantially depolarised resting membrane potentials. In human platelets, margatoxin reduced the $P2X_1$- and thromboxaneA_2 receptor-evoked $[Ca^{2+}]_i$ increases and delayed the onset of store-operated Ca^{2+} influx. Megakaryocyte development was normal in Kv1.3$^{-/-}$ mice, but the platelet count was increased, consistent with a role of Kv1.3 in apoptosis or decreased platelet activation. We conclude that Kv1.3 forms the Kv channel of the platelet and megakaryocyte, which sets the resting membrane potential, regulates agonist-evoked Ca^{2+} increases and influences circulating platelet numbers.

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Abbreviations ACD, acid citrate dextrose; PRP, platelet-rich plasma; Kv channel, voltage-gated K^+-selective channel.

Introduction

Ion channels are a large and diverse family of transmembrane proteins that play important roles in all cell types. Their functions in the platelet are poorly understood (reviewed in Mahaut-Smith, 2004), although it is clear from patch clamp studies of platelets and megakaryocytes that the largest amplitude ionic currents are conducted through voltage-gated K^+-selective (Kv) channels (Maruyama, 1987; Kawa, 1990; Kapural *et al.* 1995; Romero & Sullivan, 1997). These channels activate on depolarisation to potentials positive to −60 mV, are steeply voltage dependent over the range −40 to −10 mV and are maximally activated at potentials above 0 mV (Maruyama, 1987). They open and close with a relatively slow timecourse and are therefore comparable to the 'delayed rectifier' K^+ channels of excitable tissues that contribute to action potential repolarisation. In the platelet, this K^+ conductance could serve to stabilise the membrane potential at rest or following influx of Ca^{2+} or Na^+ through agonist-evoked channels such as Orai1, $P2X_1$ and TRPC6 (Varga-Szabo *et al.* 2009). Voltage-dependent K^+ channels also play crucial roles in volume regulation and cell proliferation of lymphocytes (Lewis & Cahalan, 1995; Chandy *et al.* 2004). However, the molecular composition of the voltage-gated K^+ channel(s) in platelets, and their precursor cell the megakaryocyte, is unknown. Pharmacological studies (Maruyama, 1987; Kawa, 1990; Romero & Sullivan, 1997) indicate that one or more members of the Kv1 or Kv3 families could contribute, as reported for lymphocytes (Grissmer *et al.* 1992; Lewis & Cahalan, 1995). Here we show for the first time that the voltage-gated K^+ channel of the platelet and megakaryocyte is formed by Kv1.3 subunits, with no

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evidence for a significant contribution from K^+ channel subunits of other Kv families. We also show that the channel is not essential for megakaryocyte development, but that it influences the number of circulating platelets and promotes agonist-evoked increases in intracellular Ca^{2+}, a key second messenger during platelet-dependent thrombosis.

Methods

Materials and salines

Standard external saline contained (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 D-glucose, pH 7.35 with NaOH. $CaCl_2$ was omitted for nominally Ca^{2+}-free saline. The patch pipette saline contained (in mM): 150 KCl, 2 $MgCl_2$, 10 Hepes, 10 D-glucose, pH 7.2 with KOH. Acid citrate dextrose (ACD) contained (in mM): 85 trisodium citrate, 78 citric acid, 111 glucose. Fura-2 was from Molecular Probes-Invitrogen (the Netherlands). Margatoxin and apyrase (type VII) were from Sigma (Poole, Dorset, UK).

Cell source and preparation

Marrow was removed from the femoral and tibial marrow of C57/bl6 or Kv1.3$^{-/-}$ mice by flushing with standard saline containing 0.32 U ml^{-1} apyrase. For immunohistochemical studies, clumps of marrow were immediately frozen in Tissue-Tek® O.C.T.™ compound (Sakura, the Netherlands). For electrophysiological recordings, marrow was gently triturated to disperse the cells and maintained on a rotor at room temperature for use within 12 h. The generation of Kv1.3$^{-/-}$ mice (C57bl/6 background) has been described previously (Koni *et al.* 2003); control mice (bred in-house at the University of Leicester or from Charles River, UK) were matched for age and sex. For studies of human platelets, standard phlebotomy techniques were used to draw blood from informed, consenting donors according to a protocol approved by the local ethics committees of the University of Leicester and the University of Lund. For intracellular Ca^{2+} measurements, blood was anti-coagulated with ACD, platelet-rich plasma (PRP) prepared by centrifugation at 700 *g* for 5 min and washed platelet suspensions prepared by centrifugation at 350 *g* for 20 min. Platelets were treated with aspirin (100 μM) and apyrase (0.32 U ml^{-1}). For cDNA preparation, whole human blood was collected into ACD supplemented with 2 mM EDTA, 0.1 μM PGE_1 and 300 μM aspirin as described elsewhere (Amisten *et al.* 2008). Mouse blood was withdrawn into ACD by cardiac puncture under terminal gaseous anaesthesia according to UK Home Office guidelines. All procedures within this study were performed in accordance with ethical standards as outlined in Drummond (2009).

Intracellular Ca^{2+} measurements

Ratiometric Ca^{2+} measurements from washed suspensions of fura-2-loaded platelets were conducted as described in detail previously (Rolf *et al.* 2001) using a Cairn cuvette spectrophotometer (Cairn Research Ltd, Faversham, UK). Platelets were loaded with fura-2 by incubation of PRP with 2 μM fura-2 AM for 45 min at 37°C and initially resuspended in nominally Ca^{2+}-free saline in the presence of apyrase (0.32 U ml^{-1}). $CaCl_2$ was added to obtain external Ca^{2+} as required by the specific protocol. Fura-2 was calibrated extracellularly using a K_d of 224 nM following release by digitonin (50 μM) and determination of R_{min} and R_{max} values in Ca^{2+}-free (10 mM EGTA) or Ca^{2+}-containing (2 mM $CaCl_2$) salines at neutral pH. All fura-2 recordings were corrected for background fluorescence, determined by quenching with $MnCl_2$.

Patch clamp recordings

Conventional whole-cell patch clamp recordings were conducted using an Axopatch 200B, as described in detail elsewhere (Mahaut-Smith, 2004). Megakaryocytes were identified by their large size and multilobed nucleus (Mahaut-Smith, 2004). For voltage clamp recordings, series resistance (R_s) compensation of $\geq$70% was applied. Whole-cell capacitance, which is a quantitative measurement of the megakaryocyte platelet-generating demarcation membrane system (Mahaut-Smith *et al.* 2003), was read directly from the patch clamp amplifier following compensation of the current transients evoked by a 5 mV voltage step from −80 mV, a potential range that does not activate voltage-gated channels in these cells (Mahaut-Smith *et al.* 2003; Mahaut-Smith, 2004).

Platelet counting

Murine platelets in ACD-anticoagulated whole blood were counted by flow cytometry according to the method developed by Alugupalli *et al.* (2001). Briefly, platelets were labelled with a FITC-conjugated anti-CD41 antibody (BD Pharmingen; 1 in 300 dilution) for 1 h and counted at a final dilution of >1000 in Ca^{2+}-free (1 mM EGTA) standard platelet saline in the presence of 5.5 μm diameter fluorescent beads of known density (SPHERO ACFP-50-5, Spherotech, Lake Forest, IL, USA). Fluorescence was measured at low rate in a single channel (488 nm excitation) of a FACSCalibur flow cytometer (BD Biosciences), which provided clear separation of platelets, beads and unlabelled cells (see online Supplementary figure). The platelet count was calculated from the ratio of beads to platelets, the dilution factor, and the density of beads.

cDNA generation and quantitative PCR

Platelets were purified and cDNA was generated as described elsewhere (Amisten *et al.* 2008). qPCR was performed using Quantifast SYBR Green PCR kit and QuantiTect Primer Assays (Qiagen, Venlo, the Netherlands) in a Roto-Gene 2000 thermal cycler (Corbett Life Science, NSW, Australia) according to the manufacturers' instructions. Gene expression was calculated according to the ΔΔCt method with GAPDH as a reference (Pfaffl, 2009).

Megakaryocyte size distribution

Sections 12 μm thick were cut from clumps of marrow frozen in Tissue-Tek® O.C.T.™ compound and thaw-mounted on slides. Sections were incubated in FITC-conjugated rat antimouse CD41 monoclonal antibody (BD Pharmingen) for 1 h and washed prior to analysis on an Olympus IX81 FV1000 confocal microscope. Fluorescence images (488 nm excitation, >500 nm emission) were acquired of randomly selected 0.1 mm^2 fields of view (six from each of 7 WT and 7 Kv1.3$^{-/-}$ mice). Olympus FV1000 analysis software was used to draw around the periphery of stained cells and thus to compute megakaryocyte area.

Statistics

Statistical significance was assessed using either Student's *t* test (paired for intracellular Ca^{2+} recordings and unpaired for membrane capacitance, megakaryocyte size distribution and platelet count) or one-way analysis of variance (membrane potential). Significance is indicated at levels of 0.05 (*), 0.01 (**), 0.005 (***) or 0.001 (****).

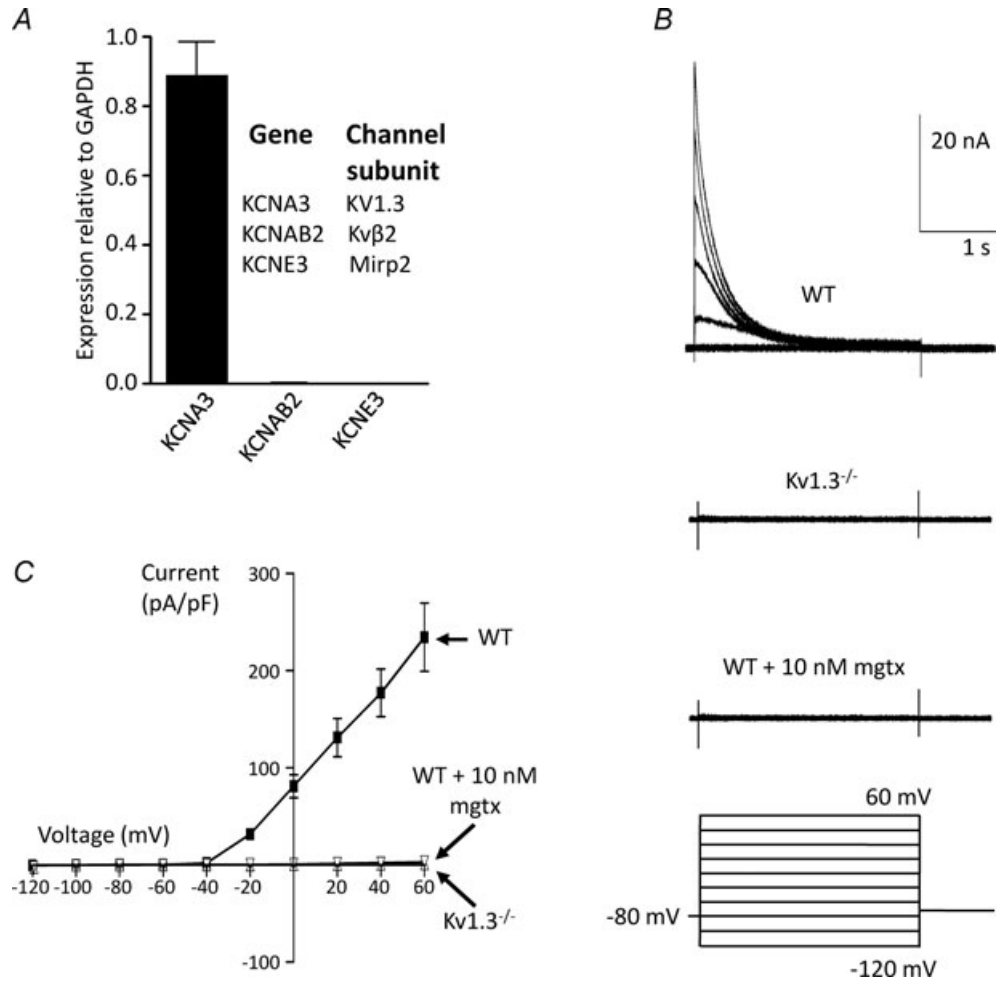

Figure 1. Kv1.3 forms the voltage-gated K^+ channel of human platelets and murine megakaryocytes
A, expression of Kv subunits relative to GAPDH in human platelets. Of all 51 known Kv subunits (see Supplementary information) only three (KCNA3, KCNAB2 and KCNE3) were detected by quantitative PCR at levels above background. *B* and *C*, typical whole-cell currents (*B*) and average peak current densities (*C*) in response to 3 s duration voltage steps from −80 mV to potentials in the range −120 to 60 mV (see voltage protocol in *B*) for wild-type (WT) megakaryocytes, Kv1.3-deficient megakaryocytes (Kv1.3$^{-/-}$) and WT megakaryocytes after 10 min exposure to 10 nM margatoxin (mgtx).

Results and Discussion

To identify components of the voltage-dependent K^+ conductance of the human platelet, we used real-time PCR to screen purified platelet cDNA for transcripts of all known α (pore-forming) and β or other ancillary subunits of the Kv family of ion channels (51 targets, see Supplementary Table 1). A single α subunit transcript was detected, for the gene *KCNA3*, which encodes Kv1.3. The only other subunits detected were for *KCNAB2* and *KCNE3* (which encode Kvβ2 and Mirp2, respectively), but both were expressed at extremely low levels (<1% of Kv1.3; Fig. 1*A*). The primary megakaryocyte is an authentic surrogate for electrophysiological studies of the small and fragile platelet (Tolhurst *et al.* 2005), therefore we used whole-cell patch clamp to investigate Kv currents of megakaryocytes from wild-type and Kv1.3-deficient mice. Using pseudophysiological internal and external salines, voltage steps from a holding potential of −80 mV activated a large transient outward current at potentials positive to about −40 mV in wild-type megakaryocytes (Fig. 1*B* top panel and Fig. 1*C*), with a mean magnitude of 11.8 ± 1.0 nA at 0 mV ($n = 14$). This voltage-gated outward current was totally absent in Kv1.3-deficient megakaryocytes (Fig. 1*B* centre panel and Fig. 1*C*), consistent with the quantitative PCR screen and implies that this channel is a homomultimer of Kv1.3. In contrast to murine lymphocytes (Koni *et al.* 2003), we failed to detect any compensatory anion currents in more than 30 recordings from Kv1.3$^{-/-}$ megakaryocytes. Margatoxin (10 nM) is a relatively selective inhibitor of Kv1.3 (Chandy *et al.* 2004; Gutman *et al.* 2005) and this blocked the outward currents (Fig. 1*B* lower panel and Fig. 1*C*), further supporting the conclusion that Kv1.3 channels mediate the voltage-gated K^+ conductance of the megakaryocyte and platelet.

In whole-cell current clamp recordings, the average membrane potential (V_m) of unstimulated megakaryocytes from wild-type mice was −46.6 ± 2 mV, $n = 7$ (Fig. 2*B*), around which regular spontaneous fluctuations of ∼2–3 mV were observed (see sample recording in Fig. 2*A*). Margatoxin (10 nM) slowly depolarised this resting potential over a timecourse that varied between cells (see, for example, Fig. 2*A*), taking between 4 and 8 min to reach a stable V_m of −14.7 ± 3 mV ($n = 7$; $P < 0.001$; Fig. 2*B*). A similar range of timecourses was observed for the block of voltage-dependent outward currents by 10 nM margatoxin in voltage clamp experiments (Fig. 1*C*). The margatoxin-induced depolarisation was slowly, and only partially, reversible (not shown), consistent with the slow wash-off (>20 min) reported previously for this toxin (Garcia-Calvo *et al.* 1993). However, the depolarisation caused by margatoxin was specifically related to block of Kv1.3 as there was no significant loss of membrane potential during the first 8 min of whole-cell recording in untreated wild-type megakaryocytes (−43.9 ± 0.9 mV, $P > 0.05$; Fig. 2*B*). The V_m of Kv1.3-deficient megakaryocytes was also substantially depolarised, on average to −10 ± 2 mV ($n = 5$; $P < 0.001$, Fig. 2*B*), further

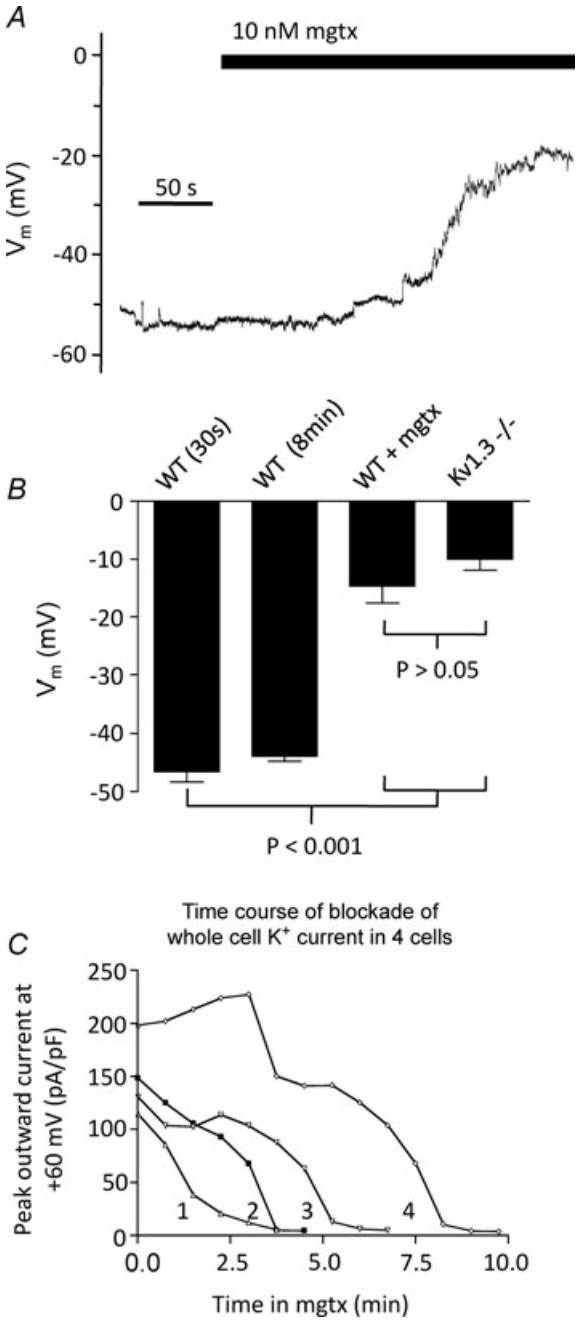

Figure 2. Kv1.3 is the major determinant of the membrane potential in mouse megakaryocytes
A, current clamp recording of membrane potential in a wild type megakaryocyte during exposure to 10 nM margatoxin (mgtx). *B*, average membrane potential in wild-type megakaryoctyes in the presence and absence of margatoxin at the times indicated after transition to whole-cell recording, and in Kv1.3$^{-/-}$ megakaryocytes. *C*, time-course of block of voltage-gated K^+ currents by 10 nM margatoxin in 4 different wild-type megakaryocytes.

supporting a major role for this channel in the resting potential.

Kv1.3 may influence platelet activation by increased agonist-evoked Ca^{2+} influx, through maintenance of the initial driving force for Ca^{2+} entry; furthermore, influx of cations will substantially depolarise the cell if counter-ion movement is not provided. In fura-2-loaded human platelets, margatoxin significantly reduced the peak Ca^{2+} increase following stimulation of $P2X_1$ (1 μM $\alpha\beta$meATP) and thromboxaneA_2 (500 nM U46619) receptors to $62 \pm 10\%$ and $76 \pm 8\%$ ($P < 0.05$) of control, respectively (Fig. 3*A*). In addition, margatoxin delayed the initial phase of store-operated Ca^{2+} influx, tested by addition of external Ca^{2+} after 10 min exposure to thapsigargin. The $[Ca^{2+}]_i$ increase stimulated after 10 s was reduced to $69 \pm 8\%$ ($P < 0.05$) of control; however, there was no significant effect on the peak Ca^{2+} increase (Fig. 3*B*). Together, these results suggest that Kv1.3 promotes early Ca^{2+} influx through multiple pathways stimulated during platelet activation, including $P2X_1$ ionotropic receptors and store-operated Ca^{2+} channels. An increase in $[Ca^{2+}]_i$ is a key signal during platelet activation (Varga-Szabo *et al.* 2009) and *in vivo* studies have demonstrated important roles for $P2X_1$ and Orai1 store-operated Ca^{2+} channel subunits in arterial thrombosis. Thus, Kv1.3 may also facilitate haemostasis and thrombosis; however, further work is required to address this issue. Direct interactions between Kv channels and integrins have been described in several cell types, including lymphocytes (Levite *et al.* 2000); thus, Kv1.3 may influence platelet aggregation and adhesion independently of effects on intracellular Ca^{2+}. Additional studies are also required to determine which other channels act as counter-ion pathways for Ca^{2+} influx in the absence of Kv1.3; however, intermediate conductance Ca^{2+}-activated K^+ channels previously reported in human platelets are a likely candidate (Mahaut-Smith, 1995).

A role for Kv channels in thymocyte development has been described (Freedman *et al.* 1995), but no differences could be detected between Kv1.3$^{-/-}$ and age/sex-matched wild-type mice in the size distribution (Fig. 4*A*; $P > 0.05$) of bone marrow megakaryocytes, suggesting that megakaryocyte development is normal. In addition, the high specific membrane capacitance of the

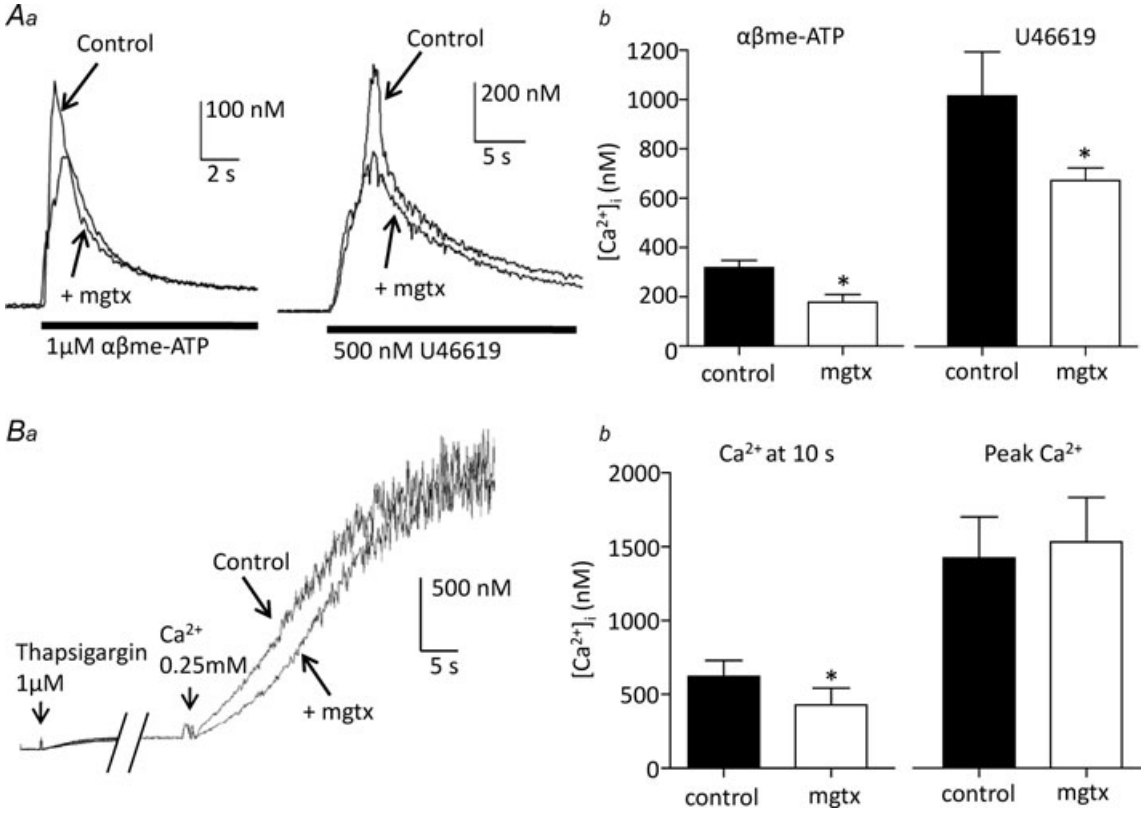

Figure 3. Role for Kv1.3 in human platelet Ca^{2+} signalling
A and *B*, typical (*a*) and average (*b*) $[Ca^{2+}]_i$ responses in fura-2-loaded washed human platelet suspensions in the presence and absence of 10 nM margatoxin (mgtx). *A*. Responses following activation of $P2X_1$ and thromboxane A_2 receptors by $\alpha\beta$meATP (1 μM) and U46619 (500 nM), respectively. *B*, store-operated $[Ca^{2+}]_i$ increases induced by addition of $CaCl_2$ (0.25 mM) after 10 min in 1 μM thapsigargin; $[Ca^{2+}]_i$ increases were measured after 10 s and at the peak of the response.

megakaryocyte, which reflects the amount of demarcation membrane system (Mahaut-Smith *et al.* 2003), was unaffected by loss of Kv1.3 ($8.19 \pm 0.24\,\mu F\,cm^{-2}$ for WT and $7.98 \pm 0.42\,\mu F\,cm^{-2}$ for Kv1.3$^{-/-}$, $P > 0.05$; Fig. 4*B*). This plasma membrane invagination system provides the additional membrane required for the process of thrombopoiesis (Schulze *et al.* 2006); therefore these data indicate that the platelet-generating capacity of the megakaryocyte is normal in Kv1.3$^{-/-}$ mice. In contrast, platelet counts were significantly increased in Kv1.3$^{-/-}$ mice compared to control mice (1.47×10^6 *vs.* $1.12 \times 10^6\,\mu l^{-1}$; $n = 7$, $P < 0.005$; Fig. 4*C*). The underlying basis of this increased platelet count is unknown and currently under investigation. One possibility is the role of mitochondrial Kv1.3 channels in apoptosis, as recently described in lymphocytes (Szabo *et al.* 2008), since platelet lifespan is controlled by an intrinsic programme of apoptosis (Mason *et al.* 2007). Alternatively, the reduced Ca^{2+} responses of Kv1.3-deficient platelets may decrease activation and thereby increase survival of circulating platelets. This is opposite to the situation in mice that express a constitutively active Stim1 mutant, whose platelets have elevated cytosolic Ca^{2+} levels and shorter circulation life-time leading to thrombocytopenia (Grosse *et al.* 2007).

In conclusion, this study demonstrates for the first time that Kv1.3 channels are responsible for the major K^+ conductance and the resting potential of the platelet. Consequently, blockade of Kv1.3 reduces Ca^{2+} entry through a number of different agonist-stimulated pathways. Kv1.3 is known to play roles in immune responses, olfaction and glucose homeostasis (Fadool *et al.* 2004; Xu *et al.* 2004; Cahalan & Chandy, 2009) and proposed as a target for treatment of multiple sclerosis (Rangaraju *et al.* 2009). The present study extends the roles of Kv1.3 to the platelet where it influences the membrane potential, Ca^{2+} responses and circulating platelet numbers.

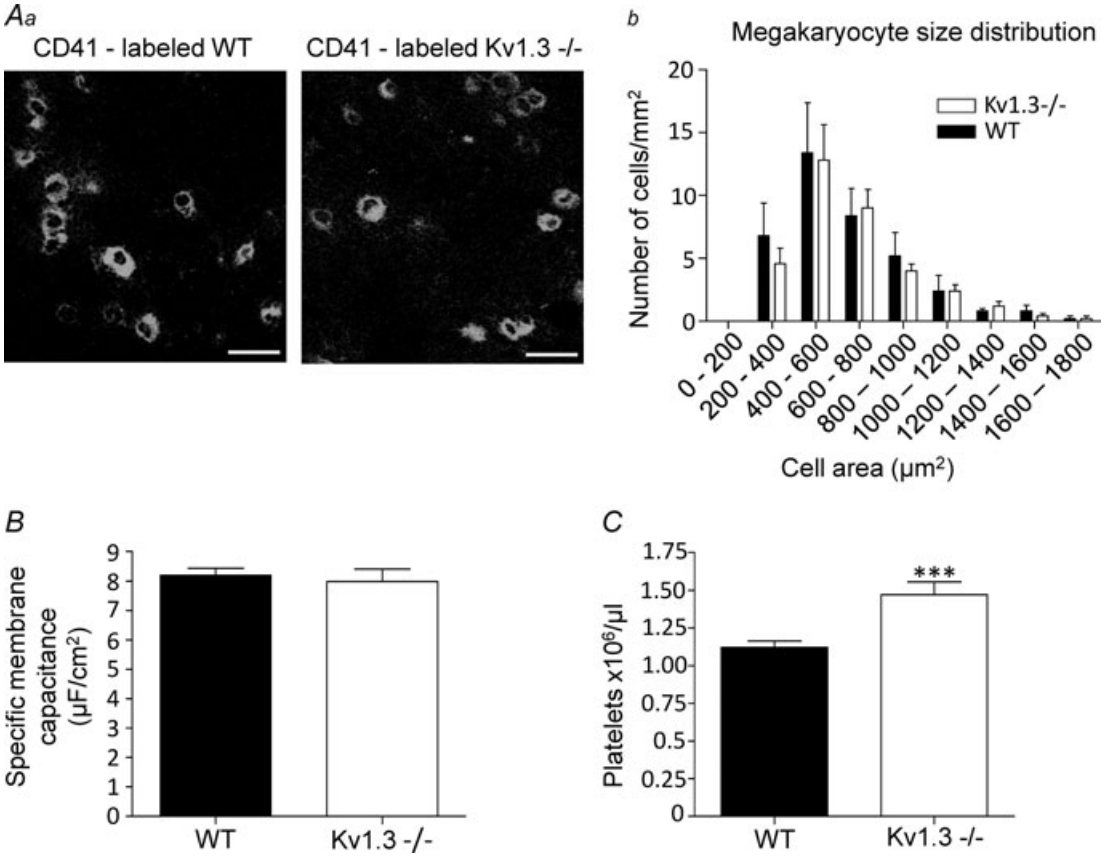

Figure 4. Role for Kv1.3 in platelet count, but not megakaryocyte development
A, megakaryocyte size distribution in marrow from wild-type and Kv1.3-deficient mice; *Aa*, sample sections stained with a FITC-conjugated anti-CD41 antibody, and *Ab*, frequency histogram of different size megakaryocytes, which showed no significant differences ($P > 0.05$) across the range of sizes. *B*, specific membrane capacitance measurement of the demarcation membrane system in Kv1.3$^{-/-}$ and wild-type megakaryocytes ($n = 23$ and 61, respectively; $P > 0.05$). *C*, platelet count in wild-type and Kv1.3-deficient mice ($n = 7$).

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Author contributions

Experiments were carried out in the Department of Cell Physiology and Pharmacology and the MRC Toxicology

Unit, University of Leicester. C.M. conducted and analysed the patch clamp, intracellular Ca^{2+} and megakaryocyte size distribution measurements; C.M., S.J., M.P.M.-S., A.H.G. and R.T.S. conducted and analysed the murine platelet counts; S.J. prepared all platelet samples except those used in qPCR studies; S.A. and D.E. conducted and analysed the platelet qPCR studies; I.D.F., S.J. and A.H.G. contributed essential discussion; I.D.F. and L.K. contributed essential materials; M.P.M.-S. designed the research and wrote the paper, which was approved by all authors.

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