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ORIGINAL ARTICLE

Amplification of human platelet activation by surface pannexin-1 channels

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Summary. *Background:* Pannexin-1 (Panx1) forms an anion-selective channel with a permeability up to ~1 kDa and represents a non-lytic, non-vesicular ATP release pathway in erythrocytes, leukocytes and neurons. Related connexin gap junction proteins have been reported in platelets; however, the expression and function of the pannexins remain unknown. *Objective:* To determine the expression and function of pannexins in human platelets, using molecular, cellular and functional techniques. *Methods:* Panx1 expression in human platelets was determined using qPCR and antibody-based techniques. Contributions of Panx1 to agonist-evoked efflux of cytoplasmic calcein, Ca^{2+} influx, ATP release and aggregation were assessed in washed platelets under conditions where the P2X1 receptor response was preserved (0.32 U mL^{-1} apyrase). Thrombus formation in whole blood was assessed *in vitro* using a shear chamber assay. Two structurally unrelated and widely used Panx1 inhibitors, probenecid and carbenoxolone, were used throughout this study, at concentrations that do not affect connexin channels. *Results: PANX1*, but not *PANX2* or *PANX3*, mRNA was detected in human platelets. Furthermore, Panx1 protein is glycosylated and present on the plasma membrane of platelets, and displays weak physical association with P2X1 receptors. Panx1 inhibition blocked thrombin-evoked efflux of calcein, and reduced Ca^{2+} influx, ATP release, platelet aggregation and thrombus formation under arterial shear rates *in vitro*. The Panx1-dependent contribution was not additive to that of P2X1 receptors. *Conclusions:* Panx1 is expressed on human platelets and amplifies Ca^{2+} influx, ATP release and aggregation through the secondary activation of P2X1 receptors. We propose that Panx1 represents a novel target for the management of arterial thrombosis.

Keywords: ATP; calcium; collagen; P2X1 receptor; pannexin 1, human.

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Introduction

Recent studies have identified the presence of connexin gap junction proteins in platelets, with important roles reported for connexins 37 and 40 [1–3]. Connexins were originally identified as the mammalian homologues of the invertebrate gap junction family, the innexins [4]. Panchin *et al*. [5] discovered a further family of three proteins with 20% sequence similarity to the innexins [4], named pannexins 1, 2 and 3 (Panx1/2/3). Expression profiling of the pannexin family has revealed that Panx1 is ubiquitously expressed whereas Panx2 is predominantly expressed in the brain and CNS, whilst Panx3 has only been detected in bone and skin [6].

Panx1 subunits are glycosylated at Asn254 before being trafficked to the plasma membrane, where they form anion-selective hexameric ion channels with a permeability up to ~1 kDa [7–11]. Panx1 opening has been shown in response to mechanical stress [11], oxygen-glucose deprivation [12], caspase cleavage [13] and an increase in $[Ca^{2+}]_i$ [14]. ATP release through Panx1 channels represents a mechanism for the activation of ATP-gated P2X1, 4 and 7 receptors in T cells [15], erythrocytes [16] and neurons [17]. The P2X1 receptor is the only member of this family of ATP-gated channels that is functional in platelets [18] and contributes to Ca^{2+} influx following stimulation by several major agonists [19,20]. *In vitro* and *in vivo* studies [21] have identified an important role for P2X1 cation channels in thrombus formation, particularly under high shear. The mechanism(s) whereby P2X1 receptors are activated following stimulation by collagen or other primary platelet agonists is incompletely understood; however, evidence suggests a predominantly autocrine mechanism of activation by released ATP [22].

Here we demonstrate that human platelets express functional Panx1 channels, which represent a novel, non-vesicular mechanism of ATP release that amplifies aggregation and Ca^{2+} influx at low concentrations of several major platelet agonists.

Materials and methods

Reagents

Collagen type I from bovine tendon was a gift from the Ethicon Corporation (Somerville, NJ, USA) and horm collagen from equine tendon was purchased from Alere (Stockport, Cheshire, UK), U46619 and thapsigargin were purchased from Calbiochem (Nottingham, Nottinghamshire, UK), and NF449 from Tocris (Bristol, UK). All other reagents were from Sigma (Gillingham, Dorset, UK). Probenecid (Prb) was prepared in normal platelet saline (NPS: in mM; 145 NaCl, 5 KCl, 1 $MgCl_2$, 10 D-glucose, 10 HEPES ~3.5 NaOH, pH 7.35) as described previously [23].

Preparation of washed human platelets

The study was approved by the University of Leicester Committee for Research Ethics concerning human subjects (non-NHS). Blood was collected into acid citrate dextrose anticoagulant (ACD; 85 mM trisodium citrate, 78 mM citric acid and 111 mM glucose) from informed, consenting donors in accordance with the Declaration of Helsinki. The blood : ACD mix (6:1) was centrifuged at 700 × *g* for 5 min. Platelet-rich plasma (PRP) was removed and treated with aspirin (100 μM) and type VII apyrase (0.32 U mL^{-1}) to preserve the P2X1 receptor response [24]. Platelets were loaded with Fura-2 or calcein by incubation with 2 μM Fura-2AM or 0.5 μM calcein-AM (Invitrogen, Paisley, UK) for 45 min at 37 °C. Washed platelets were then prepared by centrifugation at 350 x *g* for 20 min and resuspended in NPS supplemented with 0.32 U mL^{-1} apyrase. $CaCl_2$ or $MgCl_2$ (2 mM) was added to individual cuvettes for studies in the presence or nominal absence of extracellular Ca^{2+}, respectively.

Platelet aggregation and luminescence measurement of ATP secretion

Simultaneous platelet aggregation and ATP release experiments were performed at 37 °C in a model 400 lumi-aggregometer (Chronolog, Manchester, UK). Platelet suspensions were diluted 1 : 1 with NPS containing 0.32 U mL^{-1} apyrase, and 100 μg mL^{-1} fibrinogen added. ATP was measured using the CHRONO-LUME® luciferin:luciferase assay kit from Chronolog according to the manufacturer's guidelines. Luminescence values for ATP standards (30–1000 nM) were not affected by the presence of Prb or carbenoxolone (Cbx) (97.6 ± 8.6% and 95.5 ± 7.1% of control, respectively, $P > 0.05$).

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ measurements from fura-2-loaded washed platelet suspensions were performed as described previously [22].

Calcein dye efflux

Two-milliliter aliquots of calcein-loaded washed platelets (4 x 10^8 mL^{-1}) were stirred at 37 °C and 5-μL samples taken every 60 s were diluted into 0.2% formyl saline (0.85% [w/v] NaCl, 0.2% [v/v] formaldehyde) for analysis by a BD FACSCANTO flow cytometer (488 nm excitation, 530 ± 30 nm emission). The platelet population was selected based on forward and side scatter properties and included >90% of the 10 000 events counted (Fig. 3A). Dye efflux was calculated as the percentage of cells that moved out of fluorescence gate F1 (Fig. 3B).

Quantification of platelet mRNA by real-time PCR

PRP was prepared by centrifugation (180 x *g*, 20 min), and the top two-thirds were removed and treated with 2 mM EDTA, 0.1 μM PGE_1 and 0.3 mM aspirin. Residual leukocytes and erythrocytes were removed by immunomagnetic depletion [25]. mRNA was isolated using oligo-dT magnetic beads (Invitrogen) and reverse transcribed using AMV-RT (Invitrogen) as per the manufacturer's instructions. cDNA samples were screened for platelet (*GP1BB*), erythrocyte (*GYPA*) and leukocyte (*CD45*) transcripts and melt curve analysis performed to assess purity of the platelet samples (Fig. S1). Quantitative PCR (qPCR) was conducted with SYBR Green QuantiTect primer assays (Qiagen, Limburg, Netherlands) and normalized to β-actin (*ACTB*, endogenous control) expression, using the $\Delta\Delta C_T$ method [25].

Generation of stable hPanx1-His-FLAG HEK-293 cell line

Human leukocyte mRNA was reverse transcribed using AMV-RT to generate cDNA, from which the human *PANX1* (hPanx1) sequence was amplified using forward (5′-CCGGCCGGTGAACTGGGTGAAG-3′) and reverse (5′-CTCCGAGGCTCTGACAGGGCTAC-3′) primers. Restriction sites for *HindIII* and *XhoI* were introduced for ligation into pcDNA3 (Invitrogen). The final construct included a His-FLAG tag at the carboxyl terminus of Panx1 (Fig. S2). Transfection into human embryonic kidney-293 (HEK-293) cells and positive selection with geneticin (0.6 mg mL^{-1}; Invitrogen) generated a stable hPanx1-His-FLAG HEK-293 cell line.

Western blotting

Western blotting was performed as described previously [26], using antibodies listed in Table S1. For deglycosylation experiments protein lysates were treated with 750

units of PNGaseF (NEB, Ipswich, MA, USA) for 1 h at 37 °C before SDS-PAGE.

Co-immunoprecipitation

Whole platelet lysates (1 mg mL^{-1}) were centrifuged (15 700 × *g*, 10 min, 4 °C) to pellet the actin cytoskeleton. The retained supernatants were precleared for 1 h at 4 °C with protein-A (P2X1R) or protein-G (Panx1) agarose. Agarose beads were pelleted (15 700 × *g*, 2 min, 4 °C) and the supernatant retained. One microgram per milliliter of α-P2X1R or α-Panx1 antibody was added (2 h, 4 °C) before adding protein-A/G agarose to the respective samples overnight. Beads were washed three times (15 700 × *g*, 2 min, 4 °C) with 1xRIPA lysis buffer (150 mM NaCl, 50 mM Tris base, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] SDS, 1% Triton X-100, pH 8.0 with HCl) and once with 1xTBS-T (2 mM Tris base, 13.7 mM NaCl, 0.01% Tween-20, pH 7.6 with HCl) before SDS-PAGE.

Platelet membrane biotinylation

Platelet biotinylation was performed as described previously [27]. Total and streptavidin IP (surface) samples were analyzed by western blot for Panx1 and ERK1/2. Surface expression of Panx1 was quantified relative to the cytosolic protein ERK1/2, by densitometry (Image-J v1.45s, National Institutes of Health, Bethesda, MD, USA).

Immunocytochemistry

Immunocytochemistry was performed as described previously [28]. Cells were incubated with 4 μg mL^{-1} anti-Panx1 antibody (overnight, 4 °C). Following incubation with Alexafluor-647-conjugated anti-goat secondary antibody (30 min; 1 : 1000; Invitrogen), fluorescence was assessed at 635 nm excitation (650–750 nm emission) using an Olympus FV1000 confocal microscope. HEK-293 cells were fixed onto coverslips, whilst platelets were allowed to settle on uncoated glass coverslips.

Platelet adhesion and thrombus formation under flow conditions

Whole blood was collected into 40 μM PPACK (Haematologic Technologies Inc, Vermont, VT, USA), loaded with 1 μM 3,3′ dihexyloxacarbocyanine iodide ($DiOC_6$) and perfused over collagen-coated coverslips (100 μg mL^{-1}) at a shear rate of 1000 s^{-1} (parallel-plate flow chamber constructed by the Biomedical Joint Workshop, University of Leicester). Thrombi were imaged by collection of a z-series of images on an Olympus FV1000 confocal microscope at three separate fields per coverslip and analysed in Image-J. Percentage surface coverage, mean thrombus height and mean thrombus volume were calculated as described previously [29].

Statistical analysis

Platelet aggregation, ATP release and $[Ca^{2+}]_i$ measurements were normalized as a percentage of a paired control response. Data are presented as the mean ± SEM. Significance was determined using either the Student paired *t*-test or ANOVA with Bonferroni post-testing within GraphPad Prism 5 as appropriate and indicated as not significant (ns), $P < 0.05$*, $P < 0.01$** and $P < 0.001$***.

Results

Pannexin-1 is expressed on the surface of human platelets

Purified human platelet mRNA was reverse transcribed and screened by qPCR for *PANX1*, *PANX2* and *PANX3*, along with *P2RX1*, the ubiquitously expressed Na^+/K^+ ATPase (*ATP1A1*) and the platelet-specific receptor *GP1BB*. Gene expression was quantified relative to the detection level of β-actin (*ACTB*). *PANX1* mRNA was present in platelets at a similar level to *P2RX1* (Fig. 1A), whereas *PANX2* and *PANX3* were not detected (not shown). Panx1 protein expression has previously been reported in HEK-293 cells using an anti-Panx1 antibody raised against its C-terminus [30]. Immunocytochemistry using this antibody demonstrated strong fluorescence for Panx1 at the periphery of both hPanx1-His-FLAG HEK-293 cells and platelets (Fig. 1B; the inset images show that no fluorescence was detected from secondary antibody controls). Western blotting of Panx1 protein expression was performed for native and hPanx1-His-FLAG HEK-293 cells and platelets. We observed full-length Panx1 (48 kDa) in native HEK-293 cells and platelets and a 50 kDa band in hPanx1-His-FLAG HEK-293 (Fig. 1C), as predicted from a 2 kDa His-FLAG tag within the construct (Fig. S2). To further investigate Panx1 localization in platelets, we performed surface biotinylation experiments. The relative density of Panx1 to that of the cytosolic protein ERK1/2 in the total and surface lanes was determined by densitometry, which showed a 4.3-fold enhancement of Panx1:ERK1/2 for biotinylated proteins (Fig. 1D). Panx1 has previously been shown to be glycosylated on its second extracellular loop, giving rise to the fully glycosylated (48 kDa) and unglycosylated (~37 kDa) species, with only the former present at the plasma membrane [7,8]. Consistent with these previous studies, the Panx1 band in platelets (48 kDa) and hPanx1-His-FLAG HEK-293 cells (50 kDa) was reduced to ~37 kDa and ~39 kDa, respectively, after treatment with the glycosidase, PNGaseF (Fig. 1E). Together with the immunocytochemical and biotinylation data, this suggests that Panx1 is predominantly located on the surface membrane of human platelets.

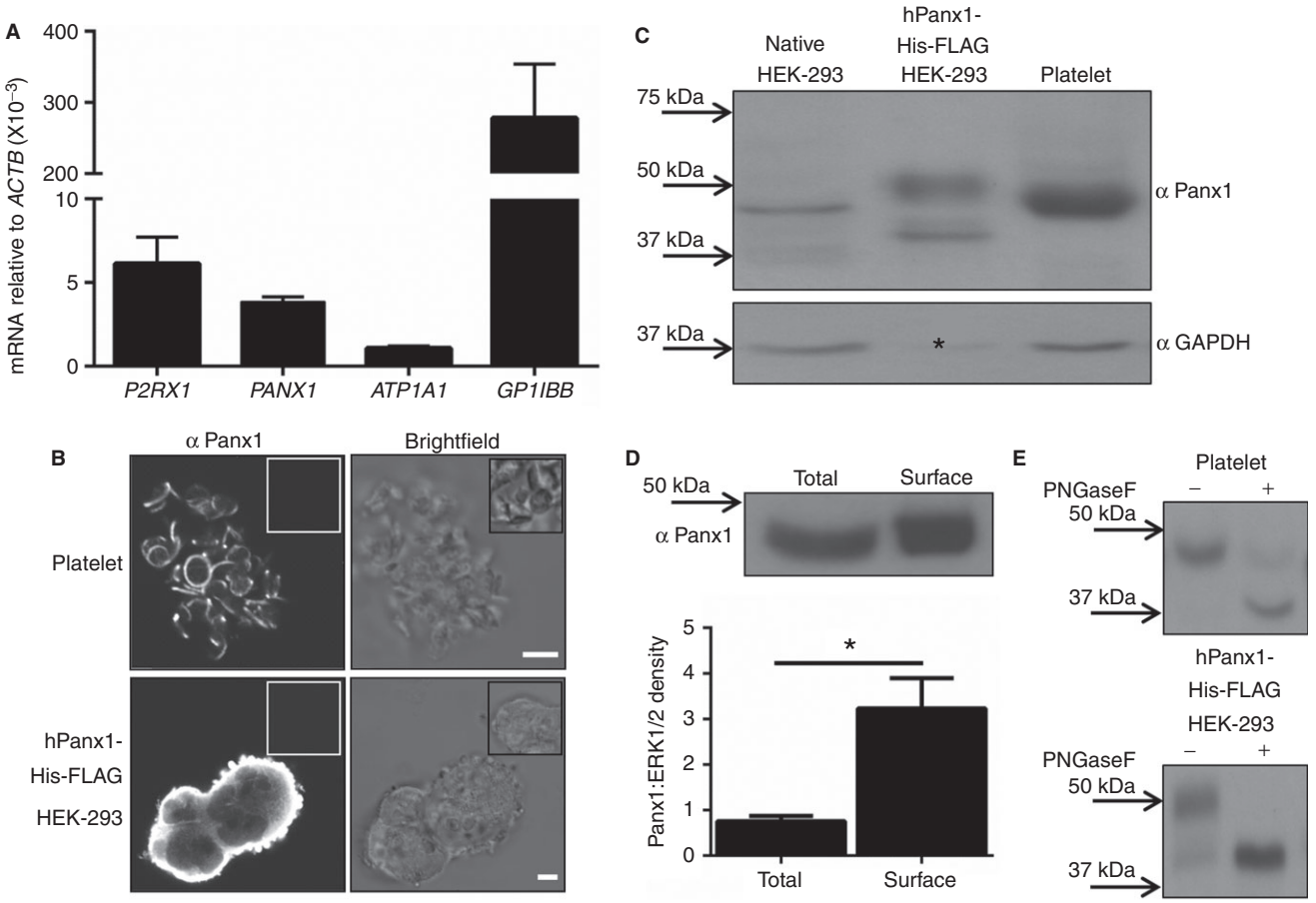

Fig. 1. Pannexin-1 is expressed on the surface of human platelets. (A) Purified platelet mRNA was reverse transcribed to cDNA and screened for transcripts encoding *P2RX1, PANX1, ATP1A1* and *GP1BB* by qPCR; expression relative to β-Actin (*ACTB*) was calculated using the $\Delta\Delta C_T$ method. (B) Immunohistochemistry shows Panx1 fluorescence in platelets and hPanx1-His-FLAG HEK-293 cells (inset, secondary antibody controls). HEK-293 cells were seeded onto 32-mm coverslips whilst platelet suspensions were added from a suspension and were allowed to settle prior to imaging. Images were acquired using an Olympus FV1000 confocal microscope using 635 nm laser excitation and 650–750 nm emission; scale bar represents 5 μm. (C) Western blot detection of Panx1 protein in native and hPanx1-His-FLAG HEK-293 cells and platelets. Relative protein loading was assessed by GAPDH (* hPanx1-His-FLAG HEK-293 cell lysates were 20-fold more dilute [0.5 μg per lane] than for platelets and native HEK-293 cells [10 μg per lane]). (D) Platelet membrane biotinylation shows Panx1 expression in the surface enriched fraction, which was further assessed by Panx1:ERK1/2 density. (E) Treatment of platelets and hPanx1-His-FLAG HEK-293 cells with PNGaseF shows a decrease in molecular weight of ~11 kDa.

Pannexin-1 modulates platelet Ca^{2+} influx evoked by multiple agonists

Locovei *et al.* [14] demonstrated that Panx1 can be activated by an increase in intracellular Ca^{2+} concentration, resulting in ATP release and amplification of Ca^{2+} responses in oocytes co-expressing Panx1 and P2Y1 receptors. We therefore examined the contribution of Panx1 to Ca^{2+} influx evoked by low concentrations of the major platelet agonists collagen (0.5 μg mL^{-1}), thrombin (0.03 U mL^{-1}), and the TXA_2 analogue U46619 (500 nM), which all rely on secondary activation of P2X1 receptors to amplify the Ca^{2+} response [20]. The collagen-evoked Ca^{2+} response was reduced to 67.3 ± 4.9% (n = 8, $P < 0.001$) and 69.1 ± 2.1% (n = 5, $P < 0.001$) of control responses by Prb and Cbx, respectively (Fig. 2A,D). These two structurally unrelated and widely used Panx1 blockers [10, 31] were applied at concentrations (100 μM Prb and 10 μM Cbx) that do not affect connexin channels reported in platelets [1,3,23,30,32]. Inhibition of Panx1 caused a similar reduction of thrombin-evoked Ca^{2+} influx, to 68.2 ± 4.5% (n = 6, $P < 0.01$) and 77.5 ± 2.8% (n = 5, $P < 0.001$) of control by Prb and Cbx, respectively (Fig. 2B,D). Finally, U46619 Ca^{2+} responses were also reduced to 79.3 ± 3.0% of control (n = 6, $P < 0.001$) by Prb and 74.7 ± 2.8% of control (n = 5, $P < 0.001$) by Cbx (Fig. 2C,D). For each agonist, there was no significant difference between the reductions caused by the two inhibitors ($P > 0.05$, Fig. 2D).

Panx1 channel opening has previously been assessed by influx or efflux of cell-impermeant indicators < 1 kDa in size [12,33]. For platelets, we used flow cytometry to measure efflux of calcein, an anionic 0.62 kDa fluorescent indicator, from the platelet cytosol (Fig. 3). Thrombin

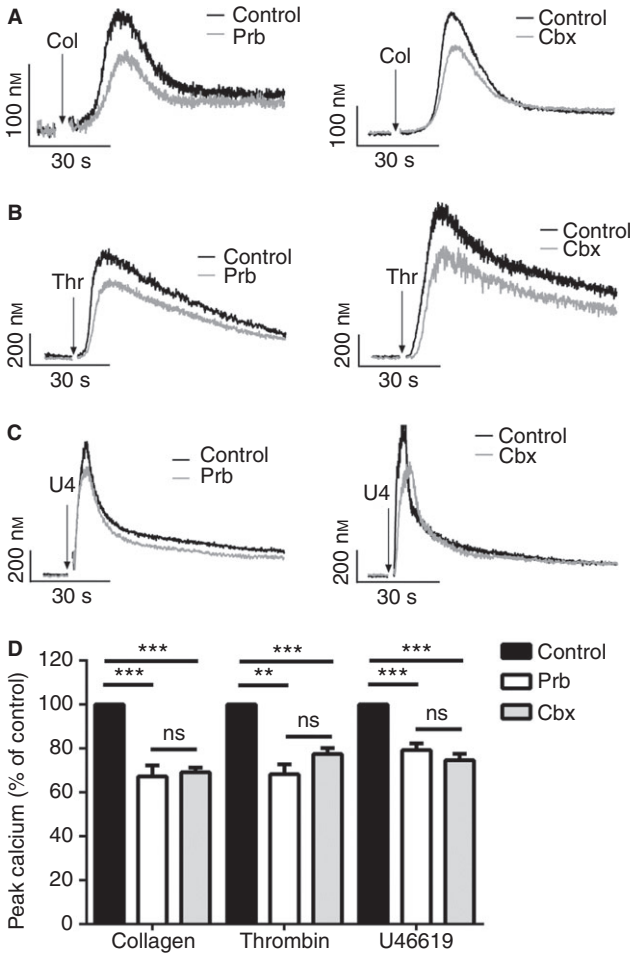

Fig. 2. Pannexin-1 modulates Ca^{2+} influx response to multiple agonists. Platelets were stimulated with 0.5 μg mL^{-1} collagen (A), 0.03 U mL^{-1} thrombin (B) or 500 nM U46619 (C) in the presence or absence of either 100 μM Prb (left panel) or 10 μM Cbx (right panel). (D) Peak Ca^{2+} responses were normalized to paired vehicle control (saline or 0.2% DMSO). Representative traces are from paired-control runs from individual donors and representative of at least five independent experiments.

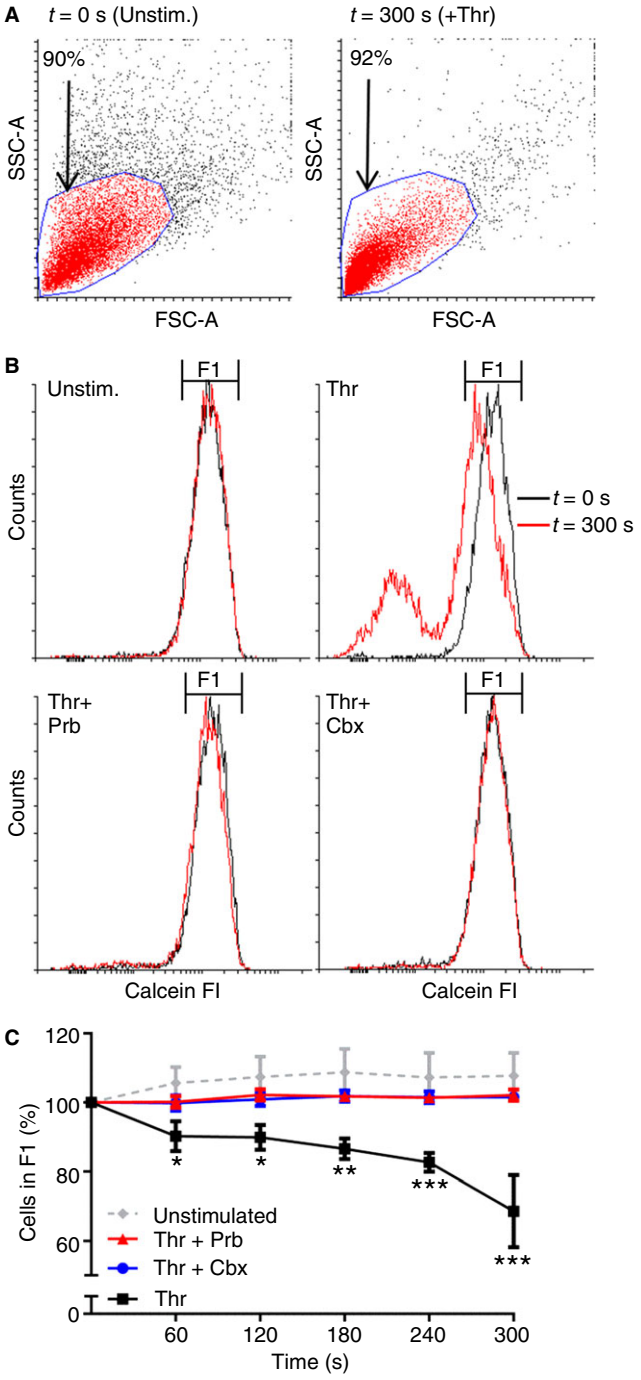

Fig. 3. Thrombin-evoked efflux of the anionic dye calcein is blocked by Panx1 inhibitors. (A) Washed platelets were selected using forward and side scatter properties to include >90% of events in resting (left) and thrombin (0.03 U mL^{-1})-stimulated (right) conditions. (B) Histograms of calcein fluorescence at t = 0 s (black) and t = 300 s (red) for either unstimulated platelets (Unstim.), platelets stimulated by thrombin (Thr), platelets stimulated by thrombin in the presence of 100 μM Prb (Thr + Prb) or platelets stimulated by thrombin in the presence of 10 μM Cbx (Thr + Cbx). (C) The percentage reduction in the proportion of cells within F1 was calculated at each 60 s time-point and is expressed relative to 0 s. Inhibitor-free runs for unstimulated and thrombin-stimulated conditions contained 0.5% DMSO as the vehicle control. Data are representative of five independent experiments.

stimulated a loss of calcein fluorescence, which was not observed in unstimulated cells and was blocked by Panx1 inhibitors (Fig. 3B). After 300 s, thrombin caused 31.4 ± 10.4% (n = 4, $P < 0.001$) of events to move from gate F1; this loss of fluorescence was completely blocked by pre-incubation with either Prb or Cbx (102.1 ± 1.7% and 101.1 ± 1.2%, respectively, compared with 107.7 ± 6.7% in unstimulated cells, $P > 0.05$; Fig. 3C). This provides further evidence that platelets express functional anion-permeable Panx1 channels on their surface, which are opened by a physiological agonist.

Panx1 could influence platelet Ca^{2+} responses by modulation of either Ca^{2+} release or a plasma membrane Ca^{2+} entry pathway. There was no significant effect of Prb or Cbx on agonist-evoked Ca^{2+} store release in the absence of external Ca^{2+} (Fig. S3), suggesting that Panx1 acts at the plasma membrane, consistent with its

predominant location (Fig. 1). Store-operated Ca^{2+} entry (SOCE) and P2X1 receptors represent the main plasma membrane Ca^{2+} entry pathways at the low agonist concentrations used in this study [20,34]. Neither Prb nor Cbx had any significant effect on SOCE activated by thapsigargin (Fig. S4) or P2X1 receptors directly activated by α,β-meATP (10 μM, Fig. S5). Our findings therefore suggest a role for Panx1 in Ca^{2+} influx across the plasma membrane, most likely by autocrine activation of P2X1 receptors by released ATP. This possibility is explored below.

Pannexin-1 amplifies collagen-evoked ATP release and platelet aggregation

To determine if Panx1 contributes to platelet activation through P2X1 receptor stimulation we assessed platelet aggregation and ATP release to 0.5 μg mL^{-1} collagen, which requires amplification by P2X1 receptors [19–22, 35]. Platelet ATP release measured by luciferin:luciferase was reduced to 18.2 ± 5.7% ($n = 4$, $P < 0.001$) and 14.7 ± 8.6% ($n = 4$, $P < 0.001$) of the paired control response by Prb and Cbx, respectively (Fig. 4A). Aggregation was also reduced by Prb and Cbx to 16.2 ± 3.3% ($n = 4$, $P < 0.001$) and 12.6 ± 2.4% ($n = 4$, $P < 0.001$) of the control response, respectively (Fig. 4A). Figure 7 shows that the collagen-evoked ATP release precedes the aggregation response. The contribution of P2X1 receptors to platelet ATP release and aggregation under the conditions of our experiments was assessed by selectively desensitizing the receptor with 0.6 μM α,β-meATP in NPS prior to addition of external Ca^{2+} and agonist [20]. Under these conditions ATP release and aggregation (Fig. 4A) to 0.5 μg mL^{-1} collagen were reduced to 25.1 ± 3.6% ($n = 4$, $P < 0.001$) and 15.3 ± 4.5% ($n = 4$, $P < 0.001$) of paired control responses, respectively. We also examined the effect of Panx1 inhibition following stimulation by high collagen concentrations, where α,β-meATP pre-addition caused smaller or no significant effects; 80.8 ± 4.3% ($n = 4$, $P < 0.05$) and 92.5 ± 5.6% ($n = 4$, $P > 0.05$), of control aggregation responses at 5 and 10 μg mL^{-1} collagen, respectively (Fig. 4B-D).

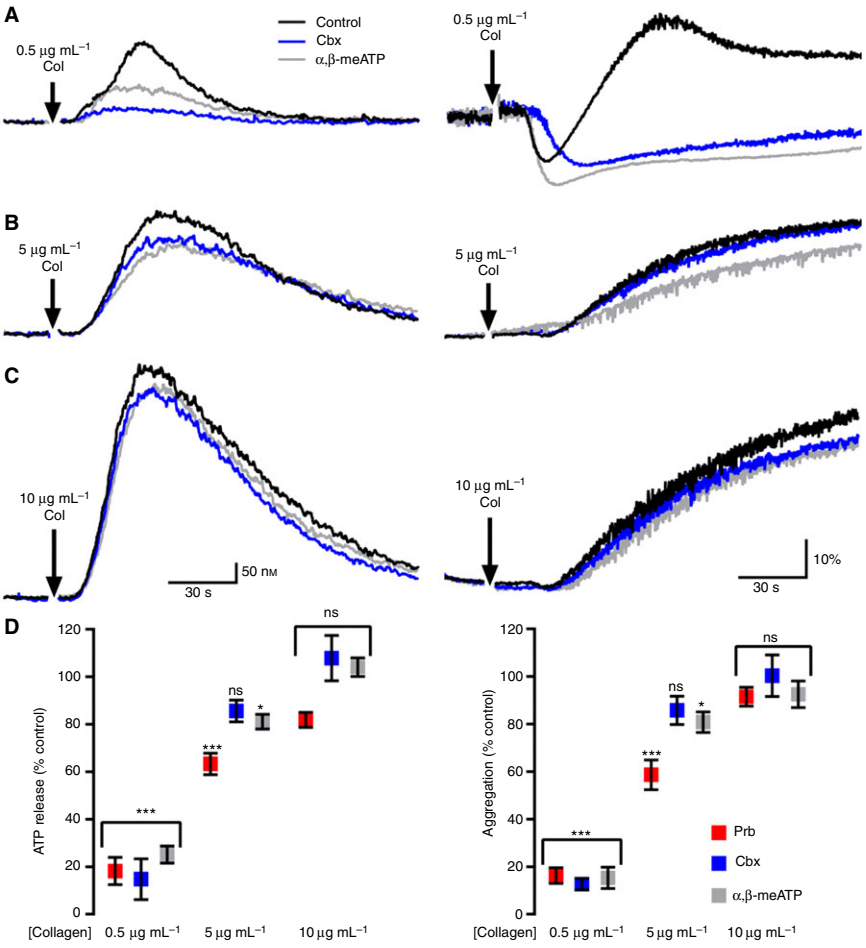

Fig. 4. Pannexin-1 amplifies ATP release and platelet aggregation at low collagen concentrations through activation of P2X1 receptors. Representative collagen-evoked platelet ATP release (left) and aggregation (right) responses to 0.5 μg mL^{-1} (A), 5 μg mL^{-1} (B) or 10 μg mL^{-1} (C) collagen. Overlaid traces show responses from control (black), 10 μM Cbx (blue) or 0.6 μM α,β-meATP (grey) treated platelets. Average responses from four independent donors are shown as a percentage of paired control response (D) for Prb (red), Cbx (blue) and α,β-meATP (grey).

Likewise, collagen-evoked ATP release was only moderately reduced by desensitization of P2X1 receptors, with peak responses of 81.1 ± 3.1% ($n = 4$, $P < 0.05$) at 5 μg mL^{-1} and no effect at 10 μg mL^{-1} (104.1 ± 3.9%, $n = 4$, $P > 0.05$). Panx1 inhibition had no significant effect on ATP release or aggregation in response to stimulation with 10 μg mL^{-1} collagen (Fig. 4C,D; $n = 4$, $P > 0.05$), and an intermediate effect was observed at 5 μg mL^{-1} collagen (Fig. 4B,D). These studies are consistent with a requirement for P2X1 receptors to observe the contribution of Panx1 in platelet functional responses. In addition, it suggests that Panx1 has a more important role in ATP release and platelet function upon stimulation by low concentrations of agonist.

At the level of Ca^{2+} mobilization, abolition of P2X1 receptor responses by pre-desensitization with 0.6 μM α,β-meATP [20], reduced the collagen-evoked Ca^{2+} increase to 33.2 ± 3.1% ($n = 5$, $P < 0.001$) of the control response, consistent with previous findings [19] (Fig. 5). No further decrease was observed following co-addition of Prb with α,β-meATP (23.3 ± 3.3%, $n = 5$, $P = 0.13$) or Cbx with α,β-meATP (21.3 ± 3.5%, $n = 5$, $P = 0.08$). We hypothesized that Panx1 may represent a route for generating localized ATP release and thus efficient, autocrine activation of P2X1 receptors. One possibility is that the two proteins are physically interacting, which we assessed by co-immunoprecipitation experiments. We observed a weak, but consistent interaction between P2X1 receptors and Panx1 in unstimulated platelet lysates (Fig. S6A). Densitometry shows a stronger signal for Panx1 when the P2X1 receptor was immunoprecipitated than vice-versa; 0.373 ± 0.027 vs. 0.152 ± 0.068 ($P < 0.001$, Fig. S6C). This stoichiometry could reflect the quaternary structure of Panx1 channels and P2X1 receptors, which form hexamers and trimers, respectively.

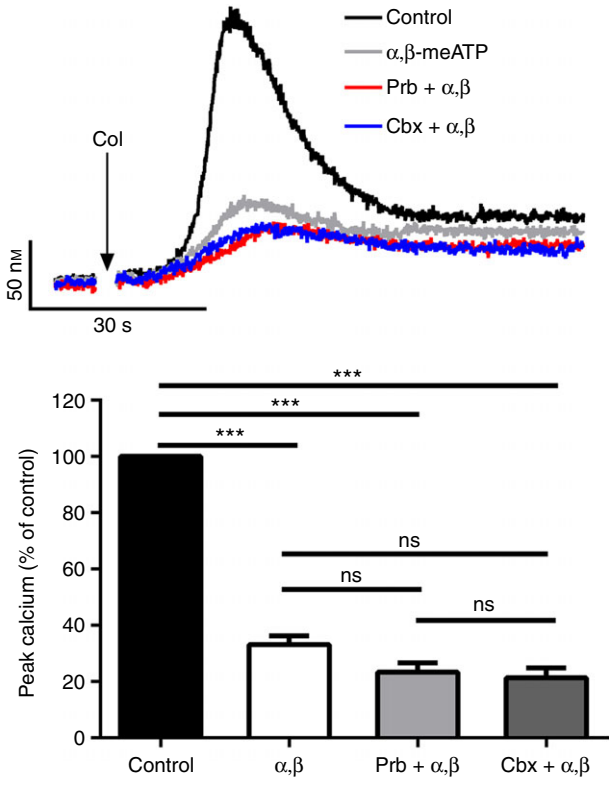

Fig. 5. Comparison of pannexin-1 and P2X1 receptor contributions to collagen-evoked Ca^{2+} responses. Upper traces: collagen-evoked (0.5 μg mL^{-1}) responses before (black trace) and after (grey trace) desensitization of P2X1 receptors with 0.6 μM α,β-meATP. Addition of 100 μM Prb (red trace) or 10 μM Cbx (blue trace) to P2X1 receptor-desensitized platelets. Lower graph: average Ca^{2+} responses expressed as a percentage of paired vehicle control; Cbx or Prb addition to P2X1 receptor-desensitized platelets did not significantly reduce the peak Ca^{2+} response compared with desensitization with α,β-meATP alone. Data are representative of five independent experiments.

Pannexin-1 is activated under arterial shear conditions in vitro

Previous studies have demonstrated Panx1 activation in response to mechanical stress [11,36]; therefore we examined its contribution to thrombus formation under flow conditions. Percentage surface coverage, thrombus volume and thrombus height were assessed by flowing whole blood over collagen-coated coverslips at an arterial shear rate of 1000 s^{-1} [29]. Surface coverage of platelets was only slightly reduced by Prb (7.5 ± 2.5%, $n = 5$, $P < 0.05$) and not affected by Cbx (2.9 ± 1.8%, $n = 4$, $P > 0.05$) (Fig. 6A–B). However, total thrombus volume (Fig. 6C) was reduced from 29 520 ± 4091 to 15 746 ± 3794 μm^3 ($n = 5$, $P < 0.01$) by Prb and from 29 914 ± 3958 to 19 886 ± 3344 μm^3 by Cbx ($n = 4$, $P < 0.05$). Furthermore, we observed a reduction in the mean thrombus height from 0.72 ± 0.10 μm to 0.40 ± 0.08 μm ($n = 5$, $P < 0.05$) by Prb and from 0.73 ± 0.10 to 0.49 ± 0.08 μm ($n = 4$, $P < 0.05$) by Cbx (Fig. 6D). These data suggest that Panx1 has a minor role in the initial adhesion events to a collagen-coated surface, but has a greater contribution to subsequent thrombus formation under flow conditions experienced in the arterial circulation. The selective P2X1 receptor antagonist NF449 (1 μM) reduced thrombus volume and height to the same extent as Panx1 inhibition (from 33 780 ± 3820 to 23 152 ± 1219 μm^3 [$n = 3$, $P < 0.05$] and 0.8240 ± 0.09 to 0.5647 ± 0.03 μm, respectively [$n = 3$, $P < 0.05$; Fig. S7]). Furthermore, co-application of NF449 with Prb did not cause a further reduction beyond the effect observed with Prb alone (20 378 ± 3212 vs. 20 594 ± 5567 μm^3; $n = 3$, $P > 0.05$) and (0.50 ± 0.08 vs. 0.50 ± 0.14 μm; $n = 3$, $P > 0.05$) for thrombus volume and height, respectively.

Discussion

This study shows for the first time that Panx1 anion-permeable channels are expressed on human platelets.

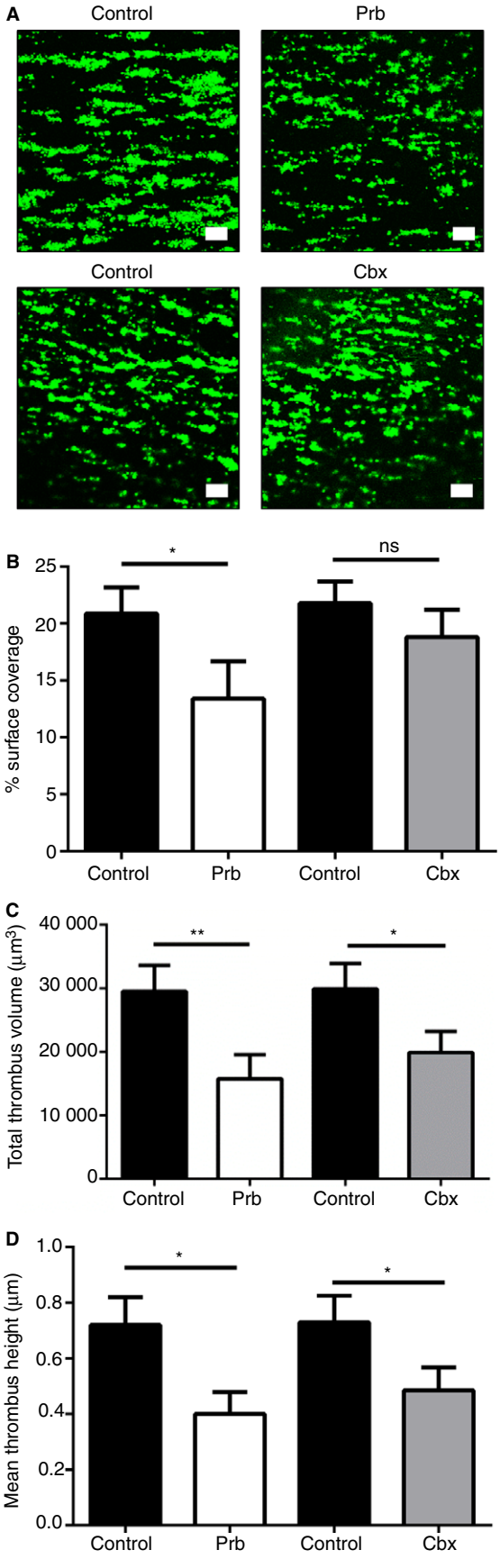

Fig. 6. Pannexin-1 contributes to thrombus formation under arterial shear conditions *in vitro*. Whole blood was treated with Prb (100 μM), Cbx (10 μM) or vehicle control and perfused over collagen-coated (100 μg mL^{-1}) coverslips at a shear rate of 1000 s^{-1}. Repre sentative images (A) of platelet adhesion and thrombus formation are shown for control, Prb- and Cbx-treated platelets. Results are expressed as the % surface coverage (B), total thrombus volume (C) and mean thrombus height (D). Scale bar represents 20 μm. Data are representative of four (Cbx) or five (Prb) independent experiments.

mRNA transcripts for *PANX1*, but not other mammalian pannexin subtypes, were detected in purified populations of platelets at similar levels to those of another ion channel (P2X1 receptor), which has established roles in thrombosis. Furthermore, glycosylation, biotinylation and immunocytochemistry experiments demonstrated that platelet Panx1 protein is fully processed and present at the plasma membrane (Fig. 1). Glycosylation at the second extracellular loop of Panx1 [9] prevents opposed Panx1 channels from docking to form gap junctions [8]. Thus, it is likely that Panx1 serves a different role to that reported for connexin 37 and 40 in platelets [1–3]. It is well established that platelets secrete ATP and ADP from dense granules in a Ca^{2+} [37] and PKC- [38] dependent manner, resulting in important amplification of platelet responses through P2X and P2Y receptors. Panx1 is known to serve as an ATP release pathway [15–17]; this channel therefore represents a novel pathway for ATP release from the platelet cytosol. Consistent with this suggestion, thrombin stimulated efflux of calcein, an anion permeable through Panx1 channels [12,33] and only slightly larger than ATP, which was blocked by Panx1 inhibitors (Fig. 3). Furthermore, Panx1 inhibitors reduced ATP release following stimulation by low collagen concentrations (Fig. 4A). Precisely how Panx1 is activated in platelets is presently unclear. A consensus sequence for PKC phosphorylation has been identified within its C-terminus that may influence channel gating [39]. However, the established role of PKC in secretion from dense granules prevented us from using inhibitors to assess the relative contribution of PKC to Panx1-mediated ATP release. Furthermore, although caspase-3 has been suggested previously to activate Panx1 in HEK-293 cells [13], the pan-caspase inhibitor z-VAD-fmk had no effect on platelet aggregation and ATP responses (data not shown). An increase in cytosolic Ca^{2+}, as shown in an oocyte expression system [14], remains a possible mechanism of Panx1 activation in platelets given the contribution to signaling we observed downstream of multiple receptor types.

Throughout this study we have relied upon the use of two inhibitors, which are not totally selective for Panx1 channels. However, we selected concentrations of these drugs that have been shown to block Panx1 activity in patch clamp studies and which do not affect other known targets, namely connexin hemichannels (Cbx) or anion transporters (Prb) [31,32,40,41]. Furthermore, connexin

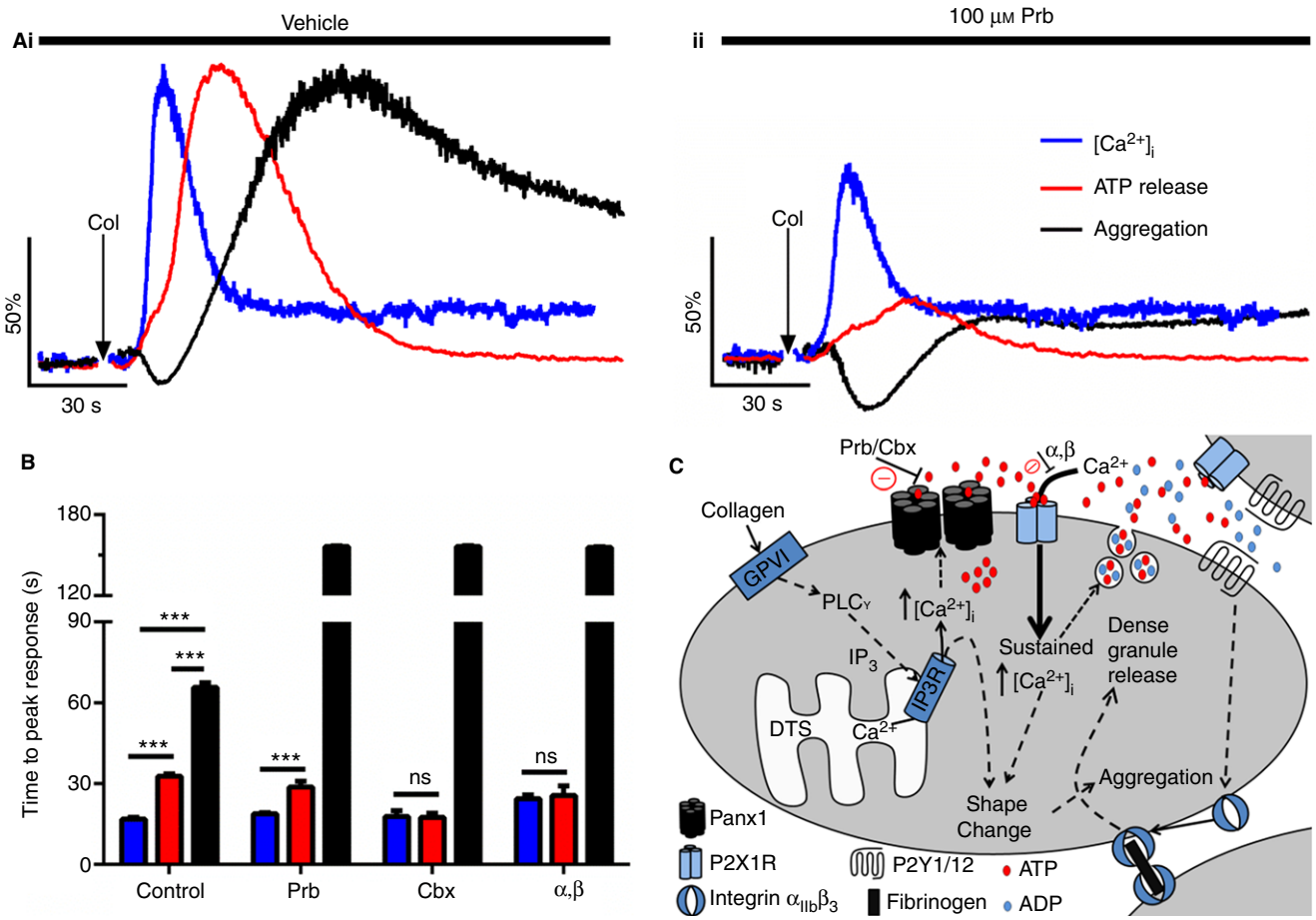

Fig. 7. A model for Panx1-dependent amplification of platelet activation based on the time-course of Ca^{2+}, ATP release and aggregation responses. Normalized collagen-evoked Ca^{2+} (blue), ATP release (red) and aggregation (black) traces overlaid from control (Ai) and 100 μM Prb-treated (Aii) platelets. (B) Mean time to peak Ca^{2+}, ATP release and aggregation response to 0.5 μg mL^{-1} collagen for control, Prb, Cbx and α,β-meATP-desensitized platelets. (C) Proposed pathway for the contribution of Panx1 to collagen-evoked platelet activation (DTS, dense tubular system).

hemichannels are closed under the conditions of our experiments (2 mM extracellular $CaCl_2$) [42–46], therefore we would not expect them to contribute to the ATP release or Ca^{2+} movements observed. Prb and Cbx also blocked calcein dye efflux (Fig. 3), consistent with previous studies of Panx1 function [12,33]. Nevertheless, it would be useful in future studies to assess the contribution of platelet Panx1 to hemostasis and thrombosis using an *in vivo* murine model and mice with Panx1-deficient platelets. Given the presence of Panx1 in a number of blood cell types [15,47], it will be crucial to develop a mouse line in which altered expression of this channel is specifically targeted to the platelet and megakaryocyte lineage.

Electrophysiological studies have demonstrated that Panx1 is an anion-selective channel [10] and is therefore unlikely to directly generate Ca^{2+} influx. Intracellular Ca^{2+} release was unaffected by Panx1 inhibitors and neither SOCE nor direct activation of P2X1 receptors were affected. In addition, at the low agonist concentrations used in this study, TRPC6 does not significantly contribute to Ca^{2+} entry [48]. Therefore, given that Prb and Cbx reduce collagen-evoked ATP release, it is likely that Prb and Cbx inhibit Ca^{2+} increases via reduction of secondary P2X1 receptor activation. The non-additive effect of Panx1 and P2X1 receptor blockade on collagen-evoked Ca^{2+} responses is consistent with this suggestion. The greater reduction of the 0.5 μg mL^{-1} collagen-evoked Ca^{2+} response by P2X1 receptor desensitization than Panx1 inhibition (Figs. 2 and 5) suggests that ATP release involves other pathways (e.g. dense granule secretion) at this low agonist concentration. Of note, ATP release through Panx1 has also been shown to activate Ca^{2+} entry through P2X1 receptors in T cells [15]. We observed only a weak physical interaction between Panx1 and P2X1 receptors as assessed by co-immunoprecipitation (Fig. S6), although the efficacy of the antibodies employed may be limited in this assay.

Considering the substantial reduction of collagen-evoked aggregation and ATP release by Prb and Cbx, Panx1 inhibition had a smaller than predicted effect on the collagen-stimulated Ca^{2+} response. However, this apparent discrepancy is likely to be explained by considering the temporal profile of collagen-evoked Ca^{2+} influx,

ATP release and aggregation under control and Panx1-inhibited conditions as shown in Fig. 7A. Collagen stimulation of platelets promotes formation of IP_3 via PLCγ [37], which leads to release of Ca^{2+} from the dense tubular system (DTS). We propose that this initial Ca^{2+} rise activates Panx1 (Fig. 7C), as described previously [14], which then releases ATP (Fig. 4) to activate P2X1 receptors, which may be co-localized (Fig. S6). Activation of P2X1 receptors amplifies the $[Ca^{2+}]_i$ increase (Fig. 5), which promotes dense granule secretion and platelet shape change. Release of ATP and ADP from dense granules amplifies the platelet activation response further through activation of P2X1 receptors and P2Y1/12 receptors, ultimately leading to platelet aggregation (Fig. 4A). We observed that platelet aggregation is dependent on nucleotide release, which in turn is dependent on a sustained increase of intracellular Ca^{2+} (Fig. 7). We therefore propose that the significant reduction in platelet aggregation in the presence of Panx1 inhibitors (Fig. 4A) is due to reduced P2X1 receptor stimulation as a result of reduced ATP release through Panx1 (Fig. 7Aii). Panx1 may also contribute to platelet P2Y receptor activation either through direct passage of ADP through Panx1 or via degradation of released ATP to ADP by ectonucleotidase activity. This pathway may represent a further mechanism for amplification of platelet activation by Panx1 and may account for the greater inhibition of aggregation than predicted from the reduction in Ca^{2+} responses. A recent study identified that Panx3 is permeable to cAMP and may therefore act as a pathway for reducing intracellular cyclic nucleotides [49], although this has not been shown for Panx1. As our platelet preparation did not include stimuli of Gs-coupled receptors or guanylate cyclase (e.g. PGI_2 or NO-donors), we would not expect to see a major contribution of cyclic nucleotide efflux to the data shown in this study.

$P2X1^{-/-}$ mice display a resistance to thrombosis in small arteries and arterioles and their platelets have a decreased propensity to develop thrombi at arterial shear rates *in vitro* [21]. Similar anti-thrombotic effects were observed *in vivo* and *in vitro* for concentrations of NF449 that selectively inhibit P2X1 receptors [50]. We also show that Panx1 inhibitors reduce thrombus development on collagen surfaces under arterial shear (Fig. 6C–D) and that NF449 had no additive effect when co-applied with Prb (Fig. S7). Taken together, these findings are consistent with a role for Panx1 in ATP release and P2X1 receptor activation under flow. In these experiments Panx1 may be activated by mechanosensation, as suggested from electrophysiological recordings of heterologously expressed channels [11,36].

In conclusion, this study demonstrates that human platelets express Panx1 channels on their surface. These represent a pathway for ATP release and thus secondary activation of P2X1 receptors, which can amplify Ca^{2+} signals and functional responses to low concentrations of major platelet agonists. Furthermore, the contribution of Panx1 to thrombus formation at arterial shear rates make it a potential candidate for the management of thrombosis.

Addendum

K. A. Taylor designed the study, performed experiments, analyzed data and wrote the paper; J. R. Wright performed experiments and analyzed data; C. Vial performed experiments and provided reagents; R. J. Evans designed the study, provided reagents and critically read the manuscript; M. P. Mahaut-Smith designed the study, analyzed data and wrote the paper. All authors have approved the final version.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Platelet cDNA is not contaminated with genetic material from other peripheral blood cells.
Fig. S2. Generation of a HEK-293 cell line stably expressing hPanx1.
Fig. S3. Pannexin-1 does not contribute to agonist-evoked Ca^{2+} release from the intracellular stores of platelets.
Fig. S4. Pannexin-1 does not contribute to SOCE in platelets.
Fig. S5. The pannexin-1 inhibitors Prb and Cbx do not directly affect P2X1 receptor-mediated Ca^{2+} influx.
Fig. S6. Pannexin-1 and P2X1 receptors display a weak physical interaction.
Fig. S7. Co-application of the P2X1 receptor antagonist NF449 and Panx1 inhibitor Prb does not further reduce thrombus volume or height.
Table S1. Antibodies used for western blotting.

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