Gap Junctions and Connexin Hemichannels Underpin Hemostasis and Thrombosis

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Background—Connexins are a widespread family of membrane proteins that assemble into hexameric hemichannels, also known as connexons. Connexons regulate membrane permeability in individual cells or couple between adjacent cells to form gap junctions and thereby provide a pathway for regulated intercellular communication. We have examined the role of connexins in platelets, blood cells that circulate in isolation but on tissue injury adhere to each other and the vessel wall to prevent blood loss and to facilitate wound repair.

Methods and Results—We report the presence of connexins in platelets, notably connexin37, and that the formation of gap junctions within platelet thrombi is required for the control of clot retraction. Inhibition of connexin function modulated a range of platelet functional responses before platelet-platelet contact and reduced laser-induced thrombosis in vivo in mice. Deletion of the Cx37 gene (Gja4) in transgenic mice reduced platelet aggregation, fibrinogen binding, granule secretion, and clot retraction, indicating an important role for connexin37 hemichannels and gap junctions in platelet thrombus function.

Conclusions—Together, these data demonstrate that platelet gap junctions and hemichannels underpin the control of hemostasis and thrombosis and represent potential therapeutic targets. (Circulation. 2012;125:2479-2491.)

Key Words: blood platelets ▪ connexin 37 ▪ gap junctions ▪ hemostasis ▪ thrombosis

Gap junctions are key mediators of intercellular communication, formed from the coupling of 2 hemichannels (or connexons) on adjacent cells. Each hemichannel results from the oligomerization of 6 connexin monomers in the cell membrane. On docking of 2 hemichannels on adjacent cells, a pore of ≈2 to 3 nm in diameter is formed that allows the bidirectional intercellular transport of small molecules up to 1000 Da. Although gap junctions have recognized roles in intercellular communication in various cell types such as nerve cells, oocytes, bone marrow stromal cells, arterial endothelial cells, and cardiac myocytes, hemichannels also act as conduits for the exchange of molecules with signaling properties between the cytoplasm and extracellular space. More than 20 connexins have been identified in mammalian cells that are able to form either homomeric or heteromeric hemichannels and gap junctions with a range of conductances and varying mechanisms of channel gating regulation.
these secreted and synthesized molecules recruit additional platelets to the first layer by stimulating platelets in a paracrine manner. At the final perpetuation stage, activation of platelets modulates the affinity of αIIbβ3, which then binds to the bivalent ligand fibrinogen and mediates platelet-platelet aggregation. Under some pathological conditions such as atherosclerotic plaque rupture, platelets are activated inappropriately, resulting in the formation of platelet aggregates within the circulation (thrombosis), a principal trigger for heart attack and stroke.

The connexin hemichannels and gap junctions have been widely studied in various cell types in which sustained cell interactions are recognized. Recent reports, however, have indicated the potential for connexins to regulate the functions of some circulating cells (monocytes and T cells). Although platelets are single circulating cells under normal conditions, on activation, the formation of a thrombus brings platelets into close proximity for a prolonged period during which they function in a coordinated manner. The sustained signaling within the thrombus regulates its stability and subsequent clot retraction, which is important for tissue repair. The present study uncovers the role of connexin hemichannels and gap junctions in platelet interactions during thrombus formation and function.

Methods

detailed methods for transcriptomic analysis, real-time quantitative polymerase chain reaction analysis, human and mouse platelet preparation, aggregation assays, dense granule secretion, immunoblotting, immunohistochemistry, transmission and scanning electron microscopy, calcium flux, flow cytometry, clot retraction, and

In vivo and in vitro thrombus formation are provided in the online-only Data Supplement.

Fluorescence Recovery After Photobleaching Measurements

Fluorescence recovery after photobleaching measurements were made on an Olympus inverted microscope with a confocal laser scanning module (Olympus FluoView1000) as described previously. Briefly, human citrated whole blood was incubated with calcein AM (2 μg/mL; Sigma Aldrich) for 20 minutes at 30°C, with exposure to gap junction blockers (100 μg/mL Gap27, 100 μM carbeneoxolone or 18-β-glycerethenic acid [18-β-GA]) or appropriate control during the final 10 minutes. The blood was then perfused over a collagen 100 μg/mL–coated coverslip in a laminar flow chamber at a shear rate of 1000 seconds^-1, and thrombi were allowed to form for 3 minutes. The unbound dye and free blood cells were washed away by perfusing platelet-poor plasma for 2 minutes. After the absence of free dye was confirmed, thrombi were selected via a 60× oil-immersion objective lens (UPLSAPO 60XO, NA 1.35, UK), and an 8-μm-diameter circular region at the center of the thrombus was exposed to high-intensity 488-nm laser light for 300 milliseconds to achieve 85% photobleach of fluorescence. Fluorescence images were continuously acquired 8 seconds before and 72 seconds after photobleach. Five thrombi were analyzed for each of 4 donors for each of the gap junction blockers. A 40-mW multiline argon laser was used for both imaging and photobleach, with outputs of 0.8% and 20%, respectively, of the 488-nm line. Average fluorescence intensities for bleached, nonbleached, and background regions were recorded for each time point. Fluorescence signals were corrected for background and steady loss of signal owing to imaging illumination and expressed as F/F0 ratios to normalize fluorescence levels (F) against starting fluorescence (F0). Fluorescence intensities were measured with Fluoview software and further analyzed with Slidebook5 software (Intelligent Imaging Innovations).

Statistical Analysis

The data obtained from fluorescence recovery after photobleaching and in vitro thrombus formation experiments in the presence and absence of various gap junction blockers were analyzed by general linear model with repeated measures through the use of the SPSS (version 17) statistical package (IBM). The data obtained from clot retraction, aggregation, fibrinogen binding, calcium mobilization, and granule secretion in human samples with gap junction blockers were analyzed simultaneously by use of nonparametric Kruskal-Wallis global statistical method, and each inhibited sample was compared with the control by use of the Dunn multiple-comparison test using GraphPad Prism (version 5.04) from GraphPad Software Inc. Data obtained from control and Cx37−/− mice were analyzed with the nonparametric Mann-Whitney test using GraphPad Prism.

Results

Presence of Connexins in Platelets

To study potential connexin expression in blood cells and megakaryocytes, we analyzed transcriptomics data obtained using an Illumina bead chip-based array by the Bloodomics Consortium. Varying levels of expression of mRNA for 16 different connexins were found in human megakaryocytes. The transcript for connexin37 (Cx37) was most highly expressed compared with the other connexins and other human blood cells (Figure 1A). Notable levels of Cx62 and Cx40 mRNAs were also detected in megakaryocytes. Quantitative polymerase chain reaction analysis with mRNA obtained from a highly purified population of human platelets also confirmed the expression of Cx37 (Figure 1B).

Connexin protein expression was investigated in human platelets. Immunoblot analysis of human platelet lysates along with positive control cell lysate (human endothelial cells) confirmed the presence of Cx37 (Figure 1C). Stimulation and activation of platelets with collagen-related peptide (CRP-XL) resulted in no change in the levels of Cx37 detected. In addition to Cx37, expression of Cx32 (Figure 1D) and Cx43 (Figure 1E) was also detected in human platelets. The identification of Cx43 in platelets is consistent with the identification of this protein in megakaryocytes. In accordance with previous reports, Cx32 and Cx43 were not detected in HeLa cell lysates. It was not possible to assess the expression of Cx62 because of the lack of specific antibodies against this protein.

The presence of connexins on the surface of platelets was confirmed by immunohistochemistry with antibodies that recognize the extracellular regions of Cx32, Cx37, Cx40, and Cx43. Washed platelets were allowed to spread on fibrinogen-coated coverslips, immunolabeled with specific antibodies, and analyzed with fluorescence microscopy. Connexins were detected on the surface of platelets (Figure 1F), an observation that was confirmed by flow cytometry (Figure 1G).

Gap Junctions in Platelets

Transmission electron microscopy was used to determine whether gap junction–like structures form between platelets. Washed human platelets were aggregated with thrombin, formed into pellets, and processed for epoxy resin embed-
The pellet was sectioned (90-nm thickness) with an ultramicrotome and stained before analysis by transmission electron microscopy. Electron micrographs show apposite membrane structures between platelets (Figure 2A) typical of gap junction–like structures well established in other cell types.1,7,29

To further explore the existence of connexins and gap junction-mediated intercellular communication between platelets in a thrombus, we used fluorescence recovery after photobleaching, which allowed the study of the migration of the fluorescent dye calcein from 1 cell to another through gap junction channels in the presence or absence of gap junction blockers after photobleaching of recipient cells.22 Fluorescence recovery was 110% in control-treated thrombi but reduced to 2% in the presence of 37,43Gap27, a connexin mimetic peptide (SRPTEKTIFII) designed to target the extracellular loops of Cx37 and Cx43, and the non–isotype-selective connexin inhibitors carbenoxolone (100 μmol/L) or 18β-GA (100 μmol/L; Figure 2B). These observations are consistent with previous fluorescence recovery after photobleaching studies of gap junctions between adipose tissue–derived mesenchymal stem cells30 and provide strong evidence for the involvement of gap junction–mediated intercellular communication between platelets within a thrombus.

We hypothesized that such communication may help regulate the coordinated response of platelets within the thrombus. After the binding of fibrinogen, the integrin αIIbβ3 transduces signals into the cell, triggering platelet spreading and, in the latter phase of thrombus formation, clot retraction.31 Such outside-in integrin signaling through αIIbβ3 may be isolated through the measurement of clot retraction in vitro.32 The effect of gap junction blockers on clot retraction was therefore measured. Platelet clots were initiated by the addition of thrombin to platelet-rich plasma in the absence or presence of gap junction blockers (100 μg/mL; Figure 1).
100 μmol/L carbenoxolone or 18β-GA), and the retraction rate of the clot was monitored over 5 hours. Initial clot formation (clot weight 15 minutes after addition of thrombin) was not altered in the presence of the gap junction blockers (Figure I in the online-only Data Supplement). Notably, clot retraction was 3-fold slower in the presence of each gap junction blocker at 90 minutes compared with control-treated samples (Figure 2C). However, after 5 hours, the clots formed in the presence of carbenoxolone and 18β-GA were retracted similar to control samples, whereas the rate of clot retraction was still slower with 37,43Gap27. These data suggest that outside-in signaling through αIIbβ3, which controls the coordinated process of clot retraction, is influenced by gap junction function.

**Gap Junction Blockers Inhibit Platelet Activation**

The effect of 37,43Gap27, carbenoxolone, and 18β-GA on platelet aggregation in response to various activators of platelet function was also explored. Different concentrations of each of the gap junction blockers were tested with a GPVI-selective ligand, CRP-XL (0.5 and 1 μg/mL), as an agonist, and the effect was recorded with optical aggregometry. Aggregation induced by a low concentration of CRP-XL was reduced at all concentrations of the gap junction blockers. Inhibition of 40%, 50%, and 75% was observed with 37,43Gap27 (100 μmol/L), carbenoxolone (100 μmol/L), and 18β-GA (100 μmol/L), respectively, when 0.5 μg/mL CRP-XL was used (Figure 3A). Lower levels of inhibition were noted with 1 μg/mL CRP-XL (Figure 3B). Aggregation was monitored over an extended period of 10 minutes, during which time inhibition was maintained (Figure IIA in the online-only Data Supplement). Similarly, 37,43Gap27-, carbenoxolone-, and 18β-GA–mediated inhibition of platelet aggregation to collagen and ADP was also observed (Figure IIC and IID in the online-only Data Supplement). A scrambled peptide (REKIITSFIPT) was used in platelet aggregation analysis as a control for 37,43Gap27 and showed no inhibitory effects (Figure III in the online-only Data Supplement).
Platelet aggregation is dependent on modulation of the conformation of $\alpha_{\text{IIb}}\beta_3$ through inside-out signaling to increase its affinity for fibrinogen binding. Thus, as a marker for inside-out signaling, fibrinogen binding was measured on the surface of the platelets by flow cytometry. Platelet-rich plasma (diluted 100-fold) was stimulated with CRP-XL (1 $\mu$g/mL) in the absence or presence of gap junction blockers for 90 seconds, and fibrinogen binding was measured. CRP-XL–stimulated fibrinogen binding was reduced significantly in the presence of gap junction blockers: 30% with Gap27 (100 $\mu$g/mL), 60% with carbenoxolone (100 $\mu$mol/L), and 70% with 18β-GA (100 $\mu$mol/L; Figure 3C). Lower concentrations of Gap27 (10 and 50 $\mu$g/mL), carbenoxolone (10 and 50 $\mu$mol/L), and 18β-GA (10 and 50 $\mu$mol/L) also showed a clear reduction in fibrinogen binding (Figure IV in the online-only Data Supplement). This illustrates the involvement of connexins in inside-out integrin signaling in platelets that results in platelet aggregation. Because this assay was performed with flow cytometry gating on the population of individual platelets, inhibition of platelet function suggests the potential role for connexin hemichannels in platelets. Functions of hemichannels in various other cell types have been reported, the inhibition of which may be achieved with gap junction blockers such as carbenoxolone, 18β-GA, and 37,43Gap27.7,8

Because aggregation was largely inhibited by the gap junction blockers at low concentrations of CRP-XL, the phosphorylation levels of various proteins involved in the GPVI pathway, which is stimulated on binding at collagen or CRP-XL, were analyzed. Platelet lysates were prepared after stimulation with CRP-XL (0.5 $\mu$g/mL; D) or thrombin (0.09 U/mL; H) in the presence or absence of gap junction blockers were analyzed by immunoblotting with anti-phosphotyrosine antibody. E, Phospho-specific antibodies against proteins involved in the glycoprotein VI (GPVI) pathway (1, resting; 2–6, platelets stimulated with CRP-XL for 90 seconds and treated with vehicle-tyrode [2], 37,43Gap27 [100 $\mu$g/mL; 3], carbenoxolone [100 $\mu$mol/L; 4], vehicle–dimethyl sulfoxide [5], and 18β-glycyrrhetinic acid [18β-GA; 100 $\mu$mol/L; 6]). Nonparametric Kruskal-Wallis global ($P$ value is shown) and Dunn multiple-comparison ($P<0.05$) tests were performed.
gesting that connexins are not involved in the control of receptor proximal events but instead can modulate the signaling further downstream, which may be shared with activation mechanisms stimulated by other agonists. This is consistent with the observed effects of gap junction blockers on the response of platelets to a range of agonists.

The effect of gap junction blockers on platelet activation stimulated by thrombin, which stimulates human platelet activation via the protease activated G-protein–coupled receptors protease-activated receptor-1 and -4,33 was measured. Thrombin-stimulated (0.09 and 0.18 U/mL) aggregation was also inhibited by each of the gap junction blockers (100 μg/mL 37,43Gap27; 100 μmol/L carbenoxolone or 18β-GA). Inhibition of 60%, 70%, and 80% with 37,43Gap27, carbenoxolone, and 18β-GA, respectively, was recorded when 0.09 U/mL thrombin was used (Figure 3F) and maintained over 10 minutes (Figure IIB in the online-only Data Supplement). Inhibition was also maintained at lower concentrations of gap junction blockers (Figure V in the online-only Data Supplement). Inhibition was markedly less pronounced with each gap junction blocker at a higher concentration of thrombin (Figure 3G). As observed with CRP-XL, the levels of thrombin-stimulated total protein tyrosine phosphorylation remained unaffected after treatment with gap junction blockers (Figure 3H). These data suggest that the connexins play a key role in platelet functional responses in both GPVI- and GPCR-stimulated activation pathways, although they do not appear to modulate early kinase-mediated signaling events.

**Platelet Connexins and Calcium Signaling**

Immediately downstream of the early signaling events, elevation of intracellular calcium levels is essential for platelet activation and thus is important in thrombus formation. Calcium mobilization plays a paramount role in various platelet functions, including reorganization of the actin cytoskeleton necessary for shape change,34 degranulation, and integrin αIIbβ3 affinity modulation.35 In platelets, a major central mechanism for the elevation of cytosolic Ca2+ is release from intracellular stores after phospholipase C–mediated generation of inositol-1,4,5-trisphosphate (IP3), which releases calcium from a dense tubular system.36 In addition, platelets express several pathways for Ca2+ influx across the plasma membrane.37 These include store-operated Ca2+ entry through Orai138–40 store-operated Ca2+ channels, ATP-gated P2X141–43 receptors, and TRPC644 channels activated by diacylglycerol or phosphatidylinositol 4,5-bisphosphate depletion.

To assess whether connexins play a role in regulating calcium mobilization, intracellular calcium levels were measured in fluo4 NW dye–loaded platelets (platelet-rich plasma) in the absence or presence of gap junction blockers (100 μg/mL 37,43Gap27, 100 μmol/L carbenoxolone) for 90 seconds after stimulation with CRP-XL (0.5 or 1 μg/mL) by spectrofluorimetry. Similar to their effects on aggregation, peak calcium levels were reduced by 25% and 40% by 37,43Gap27 and carbenoxolone, respectively, after stimulation with CRP-XL (0.5 μg/mL; Figure 4A and 4C; 18β-GA was not used in this assay because of its incompatibility with the dye, fluo4 NW). The inhibition was maintained at lower concentrations of gap junction blockers on stimulation with 0.5 μg/mL CRP-XL (Figure VIA and VIB in the online-only Data Supplement). The inhibitory effects were maintained at the higher concentrations of CRP-XL (Figure 4B and 4D). Similar experiments were performed in the presence of EGTA to block calcium influx and the formation of platelet aggregates after platelet stimulation. The overall cytosolic calcium levels were reduced compared with the levels obtained in the absence of EGTA, although the gap junction blockers maintained their ability to inhibit cytosolic calcium elevation (15% by 37,43Gap27 and 25% by carbenoxolone) stimulated by CRP-XL (Figure 4E–4H). The inhibitory effects were still observed at lower concentrations of gap junction blockers on stimulation with 0.5 μg/mL CRP-XL (Figure VIC and VID in the online-only Data Supplement). These results suggest that connexins predominantly influence release of calcium from intracellular stores in platelets, although they may also control influx of calcium across the plasma membrane. Although the exact mechanism is unknown, it is interesting to note that extracellular Ca2+ levels are known to modulate hemichannel opening in other cell types.7 Therefore, connexins could operate to promote intercellular signaling by diffusion of messengers such as IP3 and Ca2+ between platelets after contact or in enhancing membrane-membrane interactions to promote outside-in signaling, as shown for Eph kinases and ephrins,45 or indeed through sustained hemichannel function within a thrombus.

**Role of Connexins in Platelet Granule Secretion**

One way in which platelets influence the formation of thrombus is through the release of granule contents. To analyze the involvement of connexins on platelet granule secretion, both α- and dense granule secretion was assayed in the absence or presence of gap junction blockers (100 μg/mL 37,43Gap27; 100 μmol/L carbenoxolone or 18β-GA). α-Granule secretion was assessed by measuring the levels of P-selectin exposed on the surface of platelets after stimulation with CRP-XL, with the use of flow cytometry analysis in washed platelets. CRP-XL 0.5 μg/mL–stimulated α-granule secretion was reduced by 30%, 70%, and 75% with 37,43Gap27, carbenoxolone, and 18β-GA, respectively (Figure 5A). Similar levels of inhibition were seen with higher concentrations of CRP-XL (eg, 1 μg/mL; Figure 5B). Furthermore, lower concentrations of gap junction blockers inhibited the level of P-selectin exposed on the surface (Figure VIIC–VID in the online-only Data Supplement).

To determine the role of hemichannels separate from the gap junctions formed during platelet–platelet contact, the effect of the connexin blockers was also assessed on α-granule secretion under conditions that disfavor aggregation (presence of EGTA 1 mmol/L, indomethacin 10 μmol/L [to prevent the thromboxane A2 synthesis], and apyrase 2 U/mL [to prevent ATP and ADP activation of platelets]). This, combined with flow cytometry analysis, enabled their effects on individual platelets to be studied. The maintenance of single platelets was confirmed by microscopy. α-Granule secretion was reduced by 30%, 70%, and 80% with 37,43Gap27, carbenoxolone, and 18β-GA, respectively, at a low concentration of CRP-XL (0.5 μg/mL; Figure 5C), and...
the inhibition was maintained at a higher concentration of CRP-XL (1 μg/mL; Figure 5D). Similarly, the level of inhibition was clearly observed when low concentrations of gap junction blockers used (Figure VII–VIII in the online-only Data Supplement).

The effect of gap junction blockers on dense granule secretion was assessed by measuring ATP secretion after platelet activation with CRP-XL using a luciferin-luciferase luminescence assay. Platelets were activated with CRP-XL (0.5 and 1 μg/mL) in the absence or presence of gap junction blockers, and both aggregation and ATP secretion were measured simultaneously. ATP secretion was reduced by 20% in the presence of Gap27 (100 μg/mL) and 60% and 80% in the presence of carbenoxolone (100 μmol/L) and 18β-GA (100 μmol/L), respectively, at 0.5 μg/mL of CRP-XL (Figure 5E). The inhibition of dense granule secretion by gap junction blockers was still observed at higher concentration of CRP-XL, although the aggregation effects were largely overcome (Figures 3B and 5F).

Similar experiments were performed to assess the effect of gap junction blockers under conditions that disfavor aggregation (as above). ATP secretion was reduced by 25% in the presence of Gap27 (100 μg/mL) and 60% and 75% in the presence of carbenoxolone (100 μmol/L) and 18β-GA (100 μmol/L), respectively (Figure 5G). The inhibitory effects were maintained at higher concentration of CRP-XL (Figure 5H). The ability of gap junction blockers to inhibit granule secretion on activation and under conditions that disfavor aggregation indicates that connexins regulate platelet activation even in the absence of gap junction formation.

**Effect of Gap Junction Blockers on Thrombus Formation**

Given the presence of connexins on platelets, their ability to form gap junctions, and the importance of hemichannels in regulating platelet function, we speculated that connexins might make an important contribution to thrombus formation. Thus, thrombus formation was analyzed in vitro by fluorescence microscopy in whole blood under arterial flow conditions in the presence or absence of gap junction blockers. Captured images were analyzed by calculating the size and number of thrombi formed, the sum intensity of fluorescence, and the total thrombus volume. The results indicate that the size, number, and volume of thrombi were reduced by all 3 gap junction blockers (Figure 6A). The sum intensity of thrombi was reduced by 40% (at 10 minutes) with Gap27 (100 μg/mL) and 18β-GA (100 μmol/L), whereas carbenoxolone (100 μmol/L) inhibited by 65% (Figure 6B–F).

To assess the impact of gap junctions in vivo, the effect of Gap27 on arterial thrombosis was measured with a laser
injury model in mice.\textsuperscript{46} \textsuperscript{37,43}Gap27 also inhibited mouse platelet aggregation, as observed with human platelets (data not shown and Figure 3). After injury, subendothelial collagen is exposed to the blood, and thrombus formation is induced at the site of injury. Platelets were labeled with an anti-mouse GPIb\textsubscript{a} Alexa fluor-488 antibody. Thrombus formation was monitored over a period of 180 seconds at the site of injury, and the rate and size of thrombus development were analyzed by calculating the fluorescence density. Data analysis was performed for multiple thrombi obtained from 4 control- or \textsuperscript{37,43}Gap27 (100 \mu g/mL of blood)-treated mice. The initial adherence of platelets at the site of injury was relatively unaffected; thus, the initial kinetics of thrombus formation was similar in both control- and \textsuperscript{37,43}Gap27-treated animals. Overall thrombus growth, however, was reduced by \textasciitilde70\% in \textsuperscript{37,43}Gap27-treated mice (Figure 6F and 6G). Together, these data confirm the involvement of connexins in thrombus formation under in vivo conditions.

Characterization of Cx37-Deficient Mouse Platelets

Because the connexin mimetic peptide \textsuperscript{37,43}Gap27 binds Cx37 and Cx43, to further assess the specific roles of Cx37 in platelet function, we used a genetic approach and examined the function of platelets from Cx37-knockout (Cx37\textsuperscript{\textminus\textminus}) mice.\textsuperscript{47} Developmental defects in Cx37-deficient platelets were excluded through analysis of cellular and subcellular morphology and receptor expression levels. Electron microscopy analysis revealed that the morphology of platelets from Cx37\textsuperscript{\textpm} and Cx37\textsuperscript{\textminus\textminus} was indistinguishable, with \alpha- and dense granules of normal appearance and frequency (data not shown). Flow cytometry was used to measure the expression of platelet surface receptors such as \alpha\textsubscript{IIb}\beta\textsubscript{3} (Figure 7A), GPVI (Figure 7B), and GPIb (Figure 7C), which were found to be unchanged in Cx37\textsuperscript{\textminus\textminus} platelets. Similarly, the expression level of the integrin \alpha\textsubscript{2} subunit was confirmed to be normal by immunoblot analysis (Figure 7D). It is possible that deletion of the Cx37 gene (Gja4) may result in compensatory effects on the expression of other platelet connexins. The levels of other platelet connexins such as Cx32 and Cx43 (identified in this study) were also examined by immunoblot analysis. As expected, Cx37 (Figure 7E) was confirmed to be absent in Cx37\textsuperscript{\textminus\textminus} mouse platelets. Cx32 (Figure 7F) and Cx43 (Figure 7G) were detected at similar levels in platelets from Cx37\textsuperscript{\textpm} and Cx37\textsuperscript{\textminus\textminus} mice.

Effect of Cx37 Deficiency on Murine Platelet Function

Aggregation assays were performed with washed mouse platelets at the density of 2\times10\textsuperscript{9}/mL with an optical aggregometer. The aggregation of Cx37\textsuperscript{\textpm} platelets was reduced by \textasciitilde60\% at the concentration of 0.5 \mu g/mL CRP-XL (Figure 8A and 8B) compared with Cx37\textsuperscript{\textpm} platelets. At the higher concentration of CRP-XL (1 \mu g/mL), the reduction was \textasciitilde20\% (Figure 8C and 8D). These results are consistent with data obtained with human platelets in the presence of the connexin mimetic peptide inhibitor \textsuperscript{37,43}Gap27 (Figure 3A and 3B).

Fibrinogen binding on the surface of platelets (in citrated whole blood) was measured on activation by CRP-XL. This was also reduced (by \textasciitilde50\% at 0.5 \mu g/mL CRP-XL and by 30\% at 1 \mu g/mL CRP-XL) in Cx37\textsuperscript{\textminus\textminus} mice platelets compared with Cx37\textsuperscript{\textpm} platelets (Figure 8E and 8F). To

Figure 5. Role of connexins in platelet granule secretion. Granule secretion was measured by stimulating platelets with collagen-related peptide (CRP; 0.5 \mu g/mL and 1 \mu g/mL) for 90 seconds. Platelets were stimulated, and the level of P-selectin exposed on the surface was measured by flow cytometry (A and B). P-selectin exposure was also measured in the presence of EGTA (1 mmol/L; C and D). The level of P-selectin exposure with vehicle was taken as 100\%. ATP release in platelets was measured by use of luminescence-luciferase substrate in the absence (E and F) or presence (G and H) of EGTA (1 mmol/L). The level of ATP released in vehicle-treated samples was taken as 100\%. Data represent mean\pm SD (n=3). Nonparametric Kruskal-Wallis global (p value is shown) and Dunn multiple comparison (P<0.05) tests were performed.
assess the role of Cx37 on granule secretion. P-selectin was measured with flow cytometry. Granule secretion was also reduced (by \( \sim 50\% \)) at 0.5 \( \mu \text{g/mL} \) CRP-XL and 40% at 1 \( \mu \text{g/mL} \) CRP-XL in Cx37\(^{-/-}\) platelets compared with Cx37\(^{+/+}\) platelets (Figure 8G and 8H).

Given the effects of gap junction blockers on clot retraction, similar assays were also performed with Cx37\(^{-/-}\) and Cx37\(^{+/+}\) platelets. The weight of the remaining clot measured (after 5 hours) in Cx37\(^{-/-}\) was 3 times higher than in Cx37\(^{+/+}\) platelets, indicating the reduced retraction in Cx37\(^{-/-}\) platelets (Figure 8I [image shown was taken at 90 minutes]. This is consistent with the inhibitory actions of carbenoxolone, 18\(\beta\)-GA, and \(^{37,43}\)Gap27 on clot retraction in human platelet-rich plasma (Figure 2C) and point to a fundamental role for gap junction–mediated intercellular communication in the stimulation of platelet thrombus function.

**Discussion**

In the intact circulation, platelets normally circulate as individual discoid bodies but show a marked ability to adhere to each other to form a thrombus via the support of the integrin \( \alpha_{\text{IIb}}\beta_3 \) and the adhesion proteins fibrinogen and von Willebrand factor.\(^{11}\) Recent studies\(^{45}\) have indicated that sustained signaling is required to maintain thrombus stability, although little is known of how this is mediated. The contraction of the clot then ensues, a coordinated response driven by platelet integrins,\(^{21}\) that results in the shrinking of the thrombus and the drawing together of wound edges. Given that platelet function may therefore be acutely regulated while in isolation in the plasma and in concert within a thrombus, the possibility was explored that connexin hemichannels and gap junctions may be present in platelets and regulate the different phases of platelet function.
We report the expression of several members of the connexin family in human megakaryocytes and platelets in which Cx37 expression was found to be notably abundant. Consistent with the formation and function of gap junctions within the thrombus, dye transfer between cells was observed and blocked by pharmacological connexin-blocking agents, including the Cx37- and Cx43-selective mimetic peptide 37,43Gap27. The retraction of platelet-rich clots was also

Figure 7. Characterization of Cx37−/− mouse platelets. The expression levels of αIIbβ3 (A), glycoprotein (GP) VI (B), and GP Ibα (C) were analyzed by flow cytometry with citrated mouse blood. Data represent mean±SD (n=6 Cx37+/+ and Cx37−/− mice). P values calculated by the nonparametric Mann-Whitney test are shown (P<0.05). The expression level of α2 was measured by immunoblot analysis (D) using detection of 14 to 3–3ζ as a loading control. The expression of Cx37 (E), Cx32 (F), and Cx43 (G) was analyzed by immunoblots in Cx37+/+ and Cx37−/− mouse platelets. Blots are representative of 4 separate experiments.

Figure 8. Effect of Cx37 deficiency on mouse platelet function. Aggregation was recorded for 180 seconds after stimulation with collagen-related peptide (CRP-XL; 0.5 μg/mL [A] and 1 μg/mL [C]). Cumulative data (B and D) represent mean±SD. The level of aggregation obtained with Cx37+/+ was taken as 100%. The level of fibrinogen binding in Cx37+/+ and Cx37−/− was measured by flow cytometry after stimulation with CRP-XL (0.5 μg/mL [E] and 1 μg/mL [F]). Data represent mean±SD of median fluorescence intensity values. P-selectin exposure on activation by CRP-XL (0.5 μg/mL [G] and 1 μg/mL [H]) was measured with citrated mouse blood by flow cytometry. Effect of Cx37 deficiency on clot retraction was analyzed in vitro with Cx37−/− mouse platelets (I; image shown was taken at 90 minutes). Data represent mean±SD (n=4 Cx37+/+ and Cx37−/− mice) of clot weights measured after 5 hours (J). P values calculated by the nonparametric Mann-Whitney test are shown (P<0.03; n=4 Cx37+/+ and Cx37−/− mice).
reduced in the presence of these agents, strongly supporting the notion that gap junction–dependent intercellular signaling within a thrombus is required for synchronized platelet function.

Connexin blocking agents were found to inhibit a range of platelet functions, including aggregation, α-granule secretion, and fibrinogen binding, suggesting that before the association of platelets within a thrombus, hemichannels are also important for platelet function. Although calcium signaling was diminished in the presence of gap junction blockers, it is unclear whether the mode of hemichannel function may be through ion channel functionality (eg, by cooperation with Orai1,38–40 TRPC6,44 and P2X141–43) or through interaction with other components of the platelet cell machinery. As platelets become activated, the formation of initial microthrombi leading to macrothrombi may result in complex combinations of hemichannel and gap junction function, although the effect of gap junction blockers on platelets treated under conditions that disfavor platelet-platelet aggregation strongly suggests that initial platelet reactivity is modulated through hemichannels and later events within the thrombus are governed by gap junctions. The potent inhibition of both thrombus formation in vitro with human blood under arterial flow conditions and thrombosis in mice by gap junction blockers suggests that gap junctions may offer new avenues for therapeutic intervention.

Our detailed characterization of platelet function using 3 unrelated gap junction blockers in parallel point strongly to an activatory role for connexins in platelets. Because of the inability to truly inhibit the action of specific connexins, it is possible that this represents the compound actions of several connexins, each of which may exhibit different roles and regulation as heteromeric or homomeric connexons in the connexins, each of which may exhibit different roles and possible that this represents the compound actions of several gap junction blockers in parallel point strongly to avenues for therapeutic intervention.

It is interesting to reflect that recent studies implicate Cx37 in macrophages in inflammatory responses that lead to atherogenesis in susceptible mice. Indeed, a single-nucleotide polymorphism (C1019T) within the human Cx37 gene (GJA4) has been reported to be a potential prognostic marker for atherosclerosis and myocardial infarction.48,49 Given the established role of platelet-monocyte adhesion and the initiation or progression of atherosclerosis, it is tempting to speculate that the role of platelet connexin function may extend to platelet-immune cell intercellular signaling in a range of pathological conditions.

Before this study, Angelillo-Scherrer et al50 also reported the expression of Cx37 (and no other connexins) in platelets in which it was proposed to be involved in the regulation of platelet function. The prominent expression of Cx37 in human and mouse platelets is indeed consistent with our findings. In contrast to our work, however, they concluded that this connexin serves to inhibit platelet function. The reasons for this discrepancy are not entirely clear. We can conclude that platelets possess a range of connexin family members and that therefore the situation may be more complex than originally suspected, although in some cases similar experimental approaches were used. Moreover, in the above study, increased human platelet reactivity was reported with a single inhibitor, 18α-GA, which we have found to be inappropriate for the analysis of platelet function because of its ability to activate platelet function in the absence of agonists (Figure VIII in the online-only Data Supplement). Indeed, in some aggregation data (presented in the previous study), concentrations of agonists were used that in themselves did not cause aggregation in many donors, calling into question whether increased responses in the presence of 18α-GA represented increased platelet sensitivity or were due to nonspecific effects of the agent. To overcome this, in the present study, a range of pharmacological agents were used that in the absence of a platelet agonist do not cause platelet activation, allowing the study of connexin function in the presence of physiologically relevant levels of platelet activating factors. The substantial variability in response in the platelets from different donors in the previously published study was also a concern; therefore, we have presented cumulative data with appropriate statistical analysis.

Angelillo-Scherrer et al50 also reported increased aggregation responses using the platelets from Cx37−/− mice. We have scrutinized the methodologies used in both studies because the experiments reported in the present study were obtained with the same mouse line. It is possible that differences in anticoagulants used may underlie apparent discrepancies in reactivity, although ambiguity in the methodology previously presented makes this difficult to confirm. It is interesting to note that given the high agonist concentrations used in the study of Angelillo-Scherrer et al50 (eg, collagen at 5 μg/mL), very slow aggregation responses are observed, particularly for analysis in platelet-rich plasma, with no aggregation apparent until 100 seconds after stimulation. Furthermore, this is reversible, a characteristic of weak stimulation in which secretion has not been stimulated.

The analysis of hemostasis and thrombosis in Cx37−/− mice may present useful insights into the function of this protein and potentially other connexins in platelets in vivo. Indeed, Angelillo-Scherrer et al50 report a reduction in bleeding time and increased thromboembolism, although the effects of systemic deficiency of Cx37 on endothelial cell/blood vessel susceptibility to injury have not been established. It
may also be pertinent to note that Cx37 plays an important roles in lung function\textsuperscript{51,52} that may present further confounding issues in the thromboembolism model. Our analysis of thrombosis in vivo indicates that on the general inhibition of connexin function thrombi formed are less stable. It is therefore possible that thrombi formed in Cx37\textsuperscript{-/-} mice are less stable compared with Cx37\textsuperscript{+/+} mice and more susceptible to embolization, resulting in reduced survival time. It is likely that cell (platelet)-specific deletion of Cx37 and the other connexins detected in platelets will be necessary to completely unravel these inconsistencies between the studies and to examine potential differential roles of different platelet connexins.

Conclusions

Our study provides detailed evidence for a fundamental role of connexins in platelet function. The connexins, particularly Cx37, contribute to the early phases of platelet activation through formation of hemichannels. In addition, they drive thrombus formation and subsequent clot retraction after gap junction formation. Further research is required to establish the molecular nature of the signaling mechanisms through which platelet connexins exert their effects. It is possible that they contribute to calcium signaling, although additional possibilities suggested in other cell systems\textsuperscript{53} such as the dilution of cGMP levels between platelets within a thrombus and consequent reduction in inhibitory signaling may also contribute to the effects. Finally, the identification and function of connexin hemichannels and gap junctions in platelets may represent potential targets for novel antiplaque therapies.

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Disclosures

None.

References

CLINICAL PERSPECTIVE

On blood vessel injury, platelets adhere to exposed subendothelial collagens and are activated, triggering thrombus formation to prevent bleeding. Inappropriate activation of platelets under pathological conditions such as atherosclerosis results in thrombosis, which may lead to heart attack or stroke. Connexins are membrane proteins that assemble into channels (hemichannels or connexons) in the plasma membrane of selected cells, facilitating communication between the cytoplasm and external environments. Docking of connexons on neighboring cells also results in gap junctions, which mediate direct intercellular communication. Hence, hemichannels and gap junctions coordinate and synchronize the functions of various tissues and organs, eg, the heart. In the present study, we report the presence of multiple connexins in platelets. The results of this study indicate that gap junctions form between platelets in thrombi and conduct intercellular signals that promote retraction of blood clots, an important step in wound repair. Connexins were also found to regulate the activation of isolated platelets, pointing to the importance of hemichannels on platelets before thrombus formation. The results of this study indicate that gap junctions form between platelets in thrombi and conduct intercellular signals that promote retraction of blood clots, an important step in wound repair. Connexins were also found to regulate the activation of isolated platelets, pointing to the importance of hemichannels on platelets before thrombus formation. Consistent with this, the deletion of the Cx37 gene in mice resulted in reduced clot retraction and platelet activation. Inhibition of platelet connexins with pharmacological agents diminished thrombus formation in vitro in human blood and thrombotic responses in mice. Together, this study provides evidence for a fundamental role of connexin hemichannels and gap junctions in the activation of platelets and the regulation of thrombus function and suggests that connexins may represent potential avenues for the development of novel antithrombotic agents.
Gap Junctions and Connexin Hemichannels Underpin Hemostasis and Thrombosis

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SUPPLEMENTAL MATERIAL

Methods

Transcriptomics analysis

Preparation of human cell cultures and blood cells followed by transcriptomics analysis were performed by the Bloodomics Consortium as described previously. Blood was obtained from 7 healthy volunteer donors and the blood cells were isolated using an automated magnetic labelling method as described previously. Briefly, the red blood cells (RBCs) were first removed from the whole blood using gravity sedimentation through HetaSep™ starch solution (StemCell Technologies, Canada) following manufacturer’s instructions. The resulted nucleated cell-rich fraction was used to isolate granulocytes by magnetic separation using the CD66 positive selection kit (StemCell Technologies, Canada). CD4+ Th cells were isolated from peripheral blood mononuclear cells after treating the whole blood Human Monocyte Depletion cocktail RosetteSep™ (StemCell Technologies, Canada) following manufacturer’s instructions. Similarly, CD8+ Tc cells, CD14+ monocytes, CD19+ B cells and CD56+ natural killer (NK) cells were isolated from peripheral blood mononuclear cells (after removing the platelet-rich plasma) using Histopaque-1077 in an Accuspin tube (Sigma Aldrich, UK). All the cells were isolated using positive selection kits using the markers mentioned above (StemCell Technologies, Canada) to ensure high purity. To generate human megakaryocytes, CD34+ cells were isolated from umbilical cord blood and cultured for 7 days in serum-free medium supplemented with rhTpo and interleukin-1β. Gene expression analysis was performed using Illumina Human WG-6 V2 Expression BeadChips (Illumina Inc, USA) and the data were analysed using Illumina Bead-Studio software after normalising the values to the background signals. The expression levels of connexins in different cell types were clustered using CIMminer software.
**Real-time quantitative PCR analysis**

Peripheral blood was obtained from normal healthy volunteers with informed consent. Blood was collected by venepuncture, without application of a tourniquet. Platelet activation was inhibited by addition of acid-citrate dextrose (ACD) (100mM disodium citrate, 128mM D-glucose, pH 5), PGE$_1$ (0.1µM), aspirin (0.3µM), and EDTA (2µM). Platelet rich plasma (PRP) was prepared by centrifugation of anticoagulated blood for 20min at 180g at RT. PRP was incubated with CD45 beads Dynabeads® (Invitrogen, UK) for 15 minutes at RT with rotation to deplete residual leukocytes. Leukocyte-depleted PRP was then incubated with IgG Dynabeads® (Invitrogen, UK) bound with anti-CD42b (BD Pharmingen, UK), for 15 minutes at RT with rotation. Positively-selected platelets were washed in PBS and lysed in Lithium Dodecyl-Sulphate (LiDS) buffer (10mM Tris HCl (pH 7.5); 0.15M LiCl; 1mM EDTA; 0.1% LiDS). The mRNA was directly isolated using oligo(dT) Dynabeads® (Invitrogen, UK), and cDNA synthesis performed using cloned Avian Myeloblastosis Virus (AMV) Reverse Transcriptase (Invitrogen, UK) according to the manufacturer's instructions. Real-time PCR was performed using QuantiTect primer assays (Qiagen, UK) with QuantiFast SYBR green master mix (Invitrogen, UK). PCR cycling (ABI PRISM 7900HT Sequence detection System; Applied Biosystems) was performed using the parameters: 5 minutes at 95°C, 2 minutes at 50°C, followed by 40 cycles of 10s at 95°C and 1 minute at 60°C. Melting curves were performed to verify the specificity of the PCR product. Cycle threshold ($C_T$) values were normalised to expression of β-actin (ACTB).
Platelet preparation, aggregation, dense granule secretion and immunoblotting

Blood was obtained from healthy, aspirin-free, human volunteers with informed consent. Platelets were prepared and re-suspended in modified Tyrodes-HEPES buffer (134mM NaCl, 2.9mM KCl, 0.34mM Na₂HPO₄·12H₂O, 12mM NaHCO₃, 20mM HEPES and 1mM MgCl₂, pH 7.3) to the final density of 4x10⁸ cells/ml for aggregation assays as described previously. Contaminating blood cells were counted by light microscopy and were mainly erythrocytes; leucocytes were rarely encountered. Total cell contaminant level was <1 per 13000 platelets. Aggregation assays were performed (as described previously) using collagen-related peptide [(CRP-XL), a potent GPVI-selective agonist, from Prof R Farndale (University of Cambridge, UK)], thrombin (Sigma Aldrich, UK), collagen (Nycomed, Austria) or ADP (Sigma Aldrich, UK) in the presence or absence of gap junction blockers such as 37,43 Gap27 (SRPTEKTIFII) (a selective mimetic peptide which specifically blocks the extracellular loop region of Cx37 and Cx43), carbenoxolone (a synthetic derivative of glycyrrhetinic acid that non-selectively blocks gap junctions) and 18β-GA (a plant derived non-selective gap junction blocker) (Sigma Aldrich, UK). 37,43 Gap27 and carbenoxolone were dissolved in modified tyrodes-HEPES buffer and 18β-GA was dissolved in DMSO. A scrambled peptide (REKIITSFIPT) purchased from Anaspec, USA was used as a control in selected experiments where 37,43 Gap27 was used. Similarly, appropriate concentrations of tyrodes or DMSO were used as vehicle controls for carbenoxolone or 18β-GA, respectively. The concentrations of these inhibitors were chosen in the current study based on the concentration ranges used in other cell types previously.6-8 The final concentration of DMSO used in experiments was 0.01% (v/v) which is well tolerated in platelets.
ATP secretion assays were performed using luciferin-luciferase luminescence substrate (Chrono-log, USA) as described previously. SDS-PAGE and immunoblotting were performed using standard protocols as described previously using connexin specific antibodies [rabbit anti-human Cx32, 37 & 43 (In house antibodies, data not shown), mouse anti-human Cx43 & 32 (Invitrogen, UK), and rabbit anti-human Cx37 (Epitomics, USA & Invitrogen, UK)]. Rabbit anti-human GAPDH or 14-3-3ζ (Santacruz Biotechnology, USA) was used as a loading control in all the western blots. Human liver and heart lysates were obtained from Abcam, UK and HUVECs and HeLa cell lysates were prepared in house. The anti-phosphotyrosine antibody (4G10) was obtained from Millipore, USA and phospho-specific antibodies against various signalling proteins were obtained from Epitomics, USA. The secondary antibodies for immunoblotting; Cy5® goat anti-rabbit IgG and Cy3® goat anti-mouse IgG antibodies were obtained from Invitrogen, UK.

Mouse blood collection and platelet preparation

The Cx37+/+ and Cx37−/− mice were from the original colony on a C57BL6 background and the genotyping was performed as described previously. Mice were sacrificed and blood was collected immediately by cardiac puncture into a syringe containing ACD (2.5% Sodium citrate, 2% D-glucose and 1.5% citric acid) [at 1 (ACD): 9 (blood) ratio] for aggregation assays. Similarly the blood was collected into syringe containing 4% citrate [at 1 (citrate): 9 (blood) ratio] for flow cytometry and clot retraction assays. The blood was centrifuged at 203g for 8 minutes and PRP collected into fresh tubes. After addition of PGI2 (12.5ng/ml), the PRP was further centrifuged at 1028g for 5 minutes. The resultant platelet pellet was resuspended in modified tyrodes-HEPES buffer. The cell count was measured using
cellometer Auto T4 (Nexcelom Bioscience, USA). The platelets were rested for 30 minutes before aggregation assays were performed.

**Immunohistochemistry**

Washed human platelets in suspension (1x10^7 cells/ml) were allowed to spread over fibrinogen (100µg/ml) coated cover slips for 1 hour. Unbound platelets were washed and spread platelets were fixed with 3.7% (v/v) formaldehyde in modified Tyrodes-HEPES buffer for 20 minutes. After washing, cells were incubated with rabbit antibody^{12} raised against the homologous region which covers the extracellular regions of Cx32, Cx37, Cx40 and Cx43 for 1 hour followed by washing unbound antibodies 5 times with modified Tyrodes-HEPES buffer. The cells were then incubated with Alexa Fluor®-488 labelled goat anti-rabbit IgG (Invitrogen, UK) for 1 hour before washing and imaging using fluorescence microscopy.

**Transmission electron microscopy**

Human washed platelets were prepared as described previously, aggregated with 1U/ml of thrombin for 90 seconds and pelleted using a micro-centrifuge. After removing the buffer carefully, the platelets were fixed in 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 25mM HEPES (pH 7.3) overnight at 4°C. The platelet pellet was then washed twice with 25mM HEPES before secondary fixation in 1% (v/v) osmium tetroxide for 1 hour at room temperature. The platelet pellet was then washed twice with 25mM HEPES before being dehydrated through a graded acetone series. The dehydrated pellet was embedded in EPON resin according to manufacturer protocols (Agar Scientific, UK). Sections of approximately 90nm thickness were cut with a diamond knife and mounted on
formvar/ carbon TEM grids. Sections were stained with lead citrate for 10 minutes, washed in water, and observed on a Philips CM20 TEM at 80 kV and 25 K true magnification. The imaging medium was Kodak Electron image film, SO-163.

**Calcium flux**

An equal volume of PRP and Fluo-4 NW dye (Invitrogen, UK) were mixed and incubated for 30 minutes at 37°C. The platelets were then stimulated with different concentrations of CRP-XL in the absence or presence of gap junction blockers (\[^{37,43}\text{Gap27 and carbenoxolone}\]). The intensity of fluorescence was measured at 37°C for 120 seconds using an excitation wavelength of 485nm and emission at 510nm by Fluostar Optima (BMG Labtech, Germany) spectrofluorimeter. Similar experiments were performed in the presence of 1mM EGTA, 10µM indomethacin and 2U/ml apyrase to analyse the effect of gap junctions under the conditions where aggregation was disfavoured. Data were analysed by calculating the percentage of calcium released at 90 seconds.

**Flow cytometry**

Fibrinogen binding was measured using FITC labelled rabbit anti-human fibrinogen antibody (Dako UK Ltd). PRP was diluted 100-fold using HEPES buffered saline, and this (205µl) was mixed with 5µl of antibodies in the presence or absence of 15µl of gap junction blockers \[^{37,43}\text{Gap27 (100µg/ml), carbenoxolone or 18β-GA (100µM)}\] in a micro titre plate. Platelets were then stimulated with 75µl of CRP-XL to the final concentration of 1µg/ml and the data was collected at a medium flow rate (30µl/min) for 5 minutes using accuri\textsuperscript{®} C6 flow
cytometer (BD Biosciences, UK). Data were analysed by calculating the median fluorescence intensity at 90 seconds.

For P-selectin exposure, washed human platelets at a density of $5 \times 10^7$ were incubated with PE-CY$^\text{TM}5$ labelled mouse anti-human CD62P antibody (BD Biosciences, UK) for 5 minutes before stimulating with different concentrations of CRP-XL in an aggregometer in the presence or absence of gap junction blockers $^{[37,43]}$ [Gap27 (100µg/ml), carbenoxolone or 18β-GA (100µM)] for 90 seconds. The reactions were stopped by adding double the volume of 0.2% (v/v) formyl saline and the cells were analysed using flow cytometry. A total of 5000 gated events were collected and data was analysed by calculating the median fluorescence intensity (For simple comparison between vehicle and treated samples, data were converted into percentage of vehicle) of gated cells. Similar experiments were performed in the presence of 1mM EGTA, 10µM indomethacin and 2U/ml apyrase to analyse the effect of gap junctions under the conditions where aggregation disfavoured.

For identification of connexin expression on the surface of platelets, PRP diluted 10 fold in HEPES-buffered saline was incubated with various dilutions of rabbit anti-connexin antibody$^{12}$ raised against extracellular loop regions and Cy5® labelled goat anti-rabbit IgG antibody in a total of 50µl volume for 30 minutes at RT. The cells were then fixed with 450µl of 0.2% (v/v) formyl saline followed by another 10 fold dilution in 0.2% (v/v) formyl saline before analysing in the flow cytometer. A total of 5000 gated events were collected and data analysed by calculating the median fluorescence of gated cells.
Clot retraction

Human PRP was obtained as described above and rested at 30°C for 30 minutes. PRP (200µl) was mixed with 5µl of red blood cells and vehicle or gap junction blockers \[^{37,43}\text{Gap27 (100µg/ml); 100µM of carbenoxolone or 18β-GA}]\]. The final volume of this mix was made to 1ml with modified Tyrodes-HEPES buffer and incubated for 5 minutes at room temperature. The clot generation was initiated by adding thrombin (1U/ml) and a glass capillary was placed at the centre of the glass test tube, around which the clot formed. Clot weight was measured as a marker for clot retraction at different time points.

In vitro thrombus formation

In vitro thrombus formation was performed as described previously.\(^9\) Briefly, the DIOC\(_6\) (Sigma Aldrich, UK) labelled human citrated blood was pre-incubated with vehicle or gap junction blockers \[^{37,43}\text{Gap27 (100µg/ml); 100µM of carbenoxolone or 18β-GA}]\) and perfused over a collagen coated Vena8 BioChip (Cellix Ltd, Ireland) at a shear rate of 20 dynes/ cm\(^2\). Z-stack images of thrombi were obtained for every 30 seconds for up to 10 minutes using a Nikon eclipse (TE2000-U) microscope (Nikon Instruments, UK). The fluorescence intensity and thrombus volume were calculated by analysing the data using Slidebook5 software (Intelligent Imaging Innovations, USA).

In vivo thrombus formation

In vivo thrombus formation was analysed as described previously.\(^{16}\) Briefly, C57BL/6 mice were anesthetised by intraperitoneal injection of ketamine (125mg/kg), xylazine (12.5mg/kg)
and atropine (0.25mg/kg) and maintained with 5mg/kg pentobarbital as required through a jugular vein cannula. The control or 37,43 Gap27 (100µg/ml of blood) was infused into the mouse circulation 5 minutes before the injury. The cremaster muscle was exteriorized, connective tissue removed, and an incision was made to allow the muscle to be affixed as a single sheet over a glass slide. During preparation and throughout the experiment the muscle preparation was hydrated with buffer (135mM NaCl, 4.7mM KCl, 2.7mM CaCl₂, 18mM NaHCO₃, pH 7.4). Platelets were labelled with Alexa fluor®488 conjugated anti-mouse GPIbβ antibody (0.2µg/gram of mouse weight) (Emfret Analytics, Germany). The injury on the cremaster arteriole wall was induced with a Micropoint® Ablation Laser Unit (Andor technology plc, UK). Thrombus formation was observed using an Olympus BX61W1 microscope (Olympus Imaging Ltd, UK). Images were captured prior and after the injury by a Hamamatsu digital camera C9300 (Hamamatsu Photonics UK Ltd) charge-coupled device (CCD) camera in 640 x 480 format and analyzed using Slidebook5 software (Intelligent Imaging Innovations, USA).
Figures

Figure S1
Figure S2
Figure S3

A  Vehicle

B  Scrambled peptide

C  Gap27

Light transmission

Time (S)  0  90
Figure S4
Figure S5
Figure S6

A

- EGTA

Vehicle
Gap27 (10 µg/ml)
Car (10 µM)

Calcium mobilisation (%)

B

- EGTA

Vehicle
Gap27 (50 µg/ml)
Car (50 µM)

Calcium mobilisation (%)

C

+ EGTA

Vehicle
Gap27 (10 µg/ml)
Car (10 µM)

Calcium mobilisation (%)

D

+ EGTA

Vehicle
Gap27 (50 µg/ml)
Car (50 µM)

Calcium mobilisation (%)
Figure S7
Figure S8
Figure Legends

Figure S1. Effect of gap junction blockers on initial clot formation. A, Initial clot weights were measured at 15 minutes after addition of thrombin in the presence or absence of gap junction blockers (100µg/ml 37,43 Gap27, 100µM carbenoxolone or 18β-GA). Data represent mean ± S.D (n=4). The p-values calculated by non-parametric Kruskal-Wallis global statistical method and Dunn’s multiple comparison test were >0.05. B, representative image of the clots formed in the presence or absence of gap junction blockers. The clots were taken out of the tubes at 15 minutes. C, image of clots formed at 15 minutes within the tubes indicating no retraction started at this time point.

Figure S2. Gap junction blockers inhibit platelet aggregation. Aggregation performed in the presence or absence of gap junction blockers (100µg/ml 37,43 Gap27, 100µM carbenoxolone or 18β-GA) was recorded for 10 minutes following stimulation with 0.5µg/ml CRP-XL (A), 0.09U/ml thrombin (B), 1µg/ml collagen (C) and 5µM ADP (D). Traces shown are representative of four separate experiments performed using platelets from four donors.

Figure S3. Effect of scrambled peptide (control for 37,43 Gap27) on platelet aggregation. Aggregation performed in the absence (A) or presence of 100µg/ml scrambled peptide (B) or 37,43 Gap27 (C) was recorded for 90 seconds following stimulation with 0.5µg/ml CRP-XL. Traces shown are representative of four separate experiments performed using platelets from four donors.
**Figure S4. Gap junction blockers affect fibrinogen binding.** The effect of gap junction blockers (A, 10µg/ml Gap27, 10µM carbenoxolone or 18β-GA and B, 50µg/ml Gap27, 50µM carbenoxolone or 18β-GA) on fibrinogen binding was measured by flow cytometry upon stimulation with 1µg/ml CRP-XL. Data represent mean ± S.D (n=4). The level of fibrinogen binding obtained with control was taken as 100%. The p-values calculated by non-parametric Kruskal-Wallis global statistical method were <0.05 and *p<0.05, **p<0.01 and ***p<0.001 as calculated by Dunn’s multiple comparison test.

**Figure S5. Effect of gap junction blockers on thrombin induced platelet activation.** Aggregation performed in the presence or absence of gap junction blockers (A, 10µg/ml Gap27, 10µM carbenoxolone or 18β-GA and B, 50µg/ml Gap27, 50µM carbenoxolone or 18β-GA) was recorded for 90 seconds following stimulation with thrombin 0.09U/ml. Cumulative data represent mean values ± S.D (n=4). The level of aggregation obtained with control was taken as 100%. The p-values calculated by non-parametric Kruskal-Wallis global statistical method were <0.05 and *p<0.05, **p<0.01 and ***p<0.001 as calculated by Dunn’s multiple comparison test.

**Figure S6. Effect of connexins in platelet calcium mobilisation.** Calcium mobilisation was measured in Fluo4 NW dye loaded platelets by spectrofluorimetry in the presence or absence of gap junction blockers (A, 10µg/ml Gap27, 10µM carbenoxolone or 18β-GA and B, 50µg/ml Gap27, 50µM carbenoxolone or 18β-GA). Platelets were stimulated with 0.5µg/ml CRP-XL and fluorescence was measured for 90 seconds. Similar experiments were performed in the presence of EGTA (1mM) (C and D). Data represent mean ± S.D (n=4).
The calcium levels obtained at 90 seconds with control was taken as 100%. The $p$-values calculated by non-parametric Kruskal-Wallis global statistical method were $<0.05$ and $*p<0.05$, $**p<0.01$ and $***p<0.001$ as calculated by Dunn’s multiple comparison test.

**Figure S7. Role of connexins in platelet granule secretion.** Platelets were stimulated with CRP-XL (0.5µg/ml and 1 0.5µg/ml) in the presence or absence of gap junction blockers (A & B, 10µg/ml $^{37,43}$Gap27, 10µM carbenoxolone or 18β-GA and E & F, 50µg/ml $^{37,43}$Gap27, 50µM carbenoxolone or 18β-GA) and the level of P-selectin exposed on surface was measured by flow cytometry. P-selectin exposure was also measured in the presence of EGTA (1mM) (C & D and G & H). The level of P-selectin exposure with control was taken as 100%. Data represent mean ± S.D ($n=3$). The $p$-values calculated by non-parametric Kruskal-Wallis global statistical method were $<0.05$ and $*p<0.05$, $**p<0.01$ and $***p<0.001$ as calculated by Dunn’s multiple comparison test.

**Figure S8. Effect of 18α-GA on platelet activation.** 18α-GA, a gap junction blocker activates platelets on its own without addition of a platelet agonist. Different concentration of (20µM, 40µM and 100µM) this inhibitor was added to the platelets and the aggregation was monitored for 10 minutes. Indeed, 18α-GA rapidly induces the aggregation of platelets. Traces shown are representative of four separate experiments performed using platelets from four donors.
References


