Chloride channels are necessary for full platelet phosphatidylserine exposure and procoagulant activity

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Platelets enhance thrombin generation at sites of vascular injury by exposing phosphatidylserine during necrosis-like cell death. Anoctamin 6 (Ano6) is required for Ca^{2+}-dependent phosphatidylserine exposure and is defective in patients with Scott syndrome, a rare bleeding disorder. Ano6 may also form Cl^- channels, though the role of Cl^- fluxes in platelet procoagulant activity has not been explored. We found that Cl^- channel blockers or removal of extracellular Cl^- inhibited agonist-induced phosphatidylserine exposure. However, this was not due to direct inhibition of Ca^{2+}-dependent scrambling since Ca^{2+} ionophore-induced phosphatidylserine exposure was normal. This implies that the role of Ano6 in Ca^{2+-}dependent PS exposure is likely to differ from any putative function of Ano6 as a Cl^- channel. Instead, Cl^- channel blockade inhibited agonist-induced Ca^{2+} entry. Importantly, Cl^- channel blockers also prevented agonist-induced membrane hyperpolarization, resulting in depolarization. We propose that Cl^- entry through Cl^- channels is required for this hyperpolarization, maintaining the driving force for Ca^{2+} entry and triggering full phosphatidylserine exposure. This demonstrates a novel role for Cl^- channels in controlling platelet death and procoagulant activity.

The central role of blood platelets in arterial thrombosis critically depends on their ability to promote localized thrombin generation.[1] High, sustained Ca^{2+} signals trigger necrotic cell death in platelets resulting in phosphatidylserine (PS) exposure in the outer leaflet of the plasma membrane.[2] Platelet PS forms a procoagulant surface that accelerates thrombin generation. It is currently believed that sustained intracellular Ca^{2+} signalling activates a nonspecific phospholipid 'scramblase' while inhibiting an inward aminophospholipid translocase ('flippase').

Ano6 (gene *TMEM16F*) is a key regulator of calcium-dependent PS exposure.[3] Loss-of-function mutations in *TMEM16F* have been identified in two Scott syndrome patients,[3,4] who have defective calcium-dependent PS exposure in platelets, erythrocytes and B lymphocytes.[5] However, how Ano6 contributes to PS scrambling is not clear.[6] Several groups have shown that Ano6 acts as a Cl^- channel regulated by Ca^{2+} or cell volume.[7–10] However, it is not immediately clear how PS exposure could be directly regulated by Cl^- conductance.

In this study, we have used platelets to test whether Cl^- channels in principle can regulate Ca^{2+}-dependent PS exposure. We find that Cl^- channel blockers do not affect PS exposure in response to a Ca^{2+} ionophore, implying that Cl^- conductances are not necessary for platelet PS exposure. However, we also found that Cl^- channel blockers

reduce PS exposure in response to physiological platelet activators. Cl^- channels are required for plasma membrane hyperpolarization in stimulated platelets, which in turn is required for sustained Ca^{2+} signalling. These data demonstrate a novel role for Cl^- channels in platelet death and procoagulant activity.

Results

When platelets were co-stimulated by thrombin and collagen-related peptide (CRP, an agonist of GPVI (glycoprotein VI)), a substantial fraction exposed PS on their surface, as detected by annexin V binding (Figures 1a and b). To test whether Cl^- channels have a role in this, three structurally distinct Cl^- channel blockers were tested. NPPB blocks multiple Cl^- channels.[7,11] T16Ainh-A01 was identified in a screen for TMEM16A inhibitors.[12] CaCCinh-A01 blocks Ca^{2+}-activated Cl^- channels[13] and TMEM16F-dependent Cl^- currents.[7,14] Each inhibitor significantly reduced thrombin-plus-CRP-induced annexin V binding (Figure 1a), indicating that Cl^- channels are necessary for full platelet PS exposure.

Thrombin-plus-CRP-induced PS exposure was also significantly reduced in the absence of extracellular Cl^- (Figure 1b; Cl^- replaced by equimolar gluconate). This demonstrates that the effect of the Cl^- channel blockers is not a nonspecific effect on other ion channels. Together,

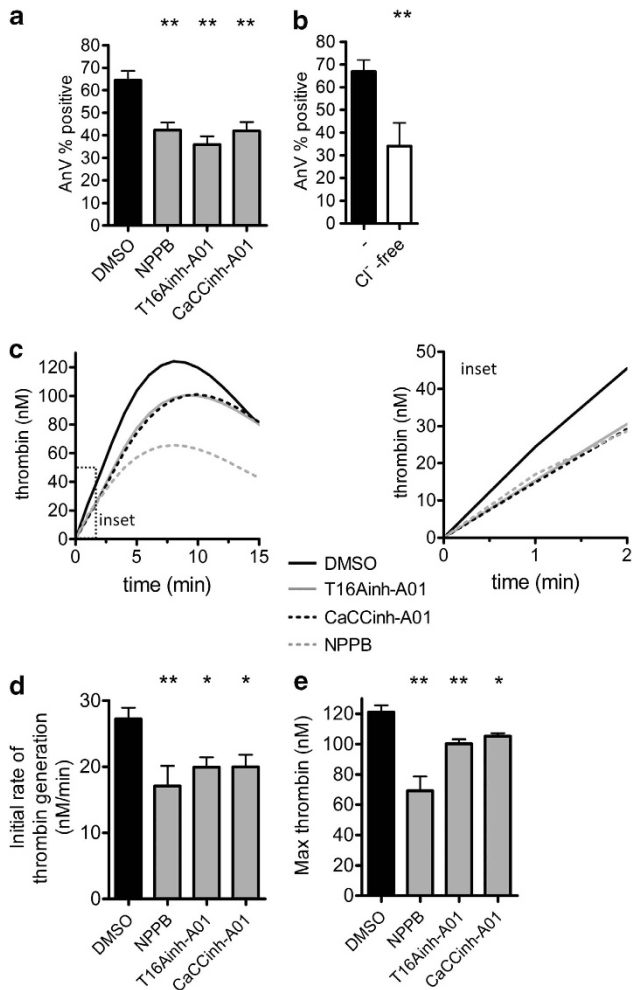**Figure 1** Chloride channel blockers partially inhibit the platelet procoagulant response. (**a**, **b**) Washed platelets were stimulated with thrombin (1 U/ml) and CRP (5 μg/ml) for 10 min and the level of phosphatidylserine exposure determined by annexin V (AnV) binding and flow cytometry. Histograms depict mean $\pm$ S.E.M. ($n = 4$–9; $P < 0.01$). (**a**) Platelets were treated with the indicated chloride channel blockers, or equivalent volume of DMSO as control, for 5 min before stimulation. (**b**) Platelets were suspended in normal NaCl-based buffer (–) or Na-gluconate-based buffer (Cl$^-$ free). (**c**–**e**) The ability of CRP-stimulated platelets to support thrombin generation in plasma was determined using a fluorescent thrombin substrate, as described in Materials and Methods. The traces from an individual experiment (**c**) are representative of four independent experiments. The right panel shows the first two minutes. (**d**, **e**) The initial rate of thrombin generations and peak thrombin generation are quantified ($n = 4$; *$P < 0.05$; **$P < 0.01$)

these data indicate that Cl$^-$ entry through Cl$^-$ channels is required for full agonist-induced PS exposure.

The role of Cl$^-$ channels in thrombin generation in platelet-rich plasma was determined by the calibrated automated thrombogram (CAT) method.[15] Platelets were stimulated with CRP, and thrombin generation was initiated with tissue factor and CaCl$_2$. Pre-treatment with Cl$^-$ channel blockers significantly reduced and slowed thrombin generation, indicating an important physiological role for these channels (Figures 1c–e).

The Cl$^-$ channel blockers did not significantly inhibit thrombin-induced P-selectin expression (a marker of

α-granule secretion). Thrombin-induced $\alpha_{IIb}\beta_3$ activation was not affected by T16Ainh-A01 and CaCCinh-A01 but was partially reduced by NPPB. Another commonly used Cl$^-$ channel blocker, DIDS, substantially reduced P-selectin expression and almost abolished $\alpha_{IIb}\beta_3$ activation, suggesting that it may have substantial off-target effects, and so was not used further in this study (Figure 2). The greater effect of NPPB on peak thrombin generation may reflect its additional effect on $\alpha_{IIb}\beta_3$ activation as signalling through $\alpha_{IIb}\beta_3$ is reported to regulate thrombin generation.[16–18]

In contrast to their inhibition of thrombin-plus-CRP-induced PS exposure, the Cl$^-$ channel blockers did not substantially inhibit PS exposure induced by the Ca^{2+} ionophore, A23187 (Figure 3). This suggests that Cl$^-$ channels may regulate Ca^{2+} signalling rather than the exposure of PS in response to a sustained increase in intracellular Ca^{2+}.

Platelet stimulation with thrombin-plus-CRP rapidly triggers a sustained increase in $[Ca^{2+}]_i$. This was substantially inhibited by the Cl$^-$ channel blockers (Figure 4a). Agonist-induced Ca^{2+} signalling in Cl$^-$-free medium was also substantially reduced (Figure 4b). Both the rapid peak increase and the area under the curve (AUC) were inhibited (Figures 4c and d). Agonist-induced Ca^{2+} signals are controlled by multiple pathways including Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} entry through Ca^{2+} channels.[19] Cl$^-$ channel blockers had no effect on thrombin-plus-CRP-induced Ca^{2+} signalling when EGTA was added to chelate extracellular Ca^{2+}, indicating that intracellular Ca^{2+} release is unaffected (Figure 4e). In contrast, divalent cation entry, as monitored by Mn^{2+} quench, was substantially inhibited (Figure 4f), suggesting that Cl$^-$ fluxes regulate Ca^{2+} entry.

Several platelet Ca^{2+} entry channels have been described including store-operated Ca^{2+} entry, involving Orai1,[20,21] and TRPC-dependent, store-independent Ca^{2+} entry.[22,23] Electrophysiological recordings have demonstrated that Orai1 and TRPCs are sensitive to changes in membrane potential.[24,25] Depolarization reduces Ca^{2+} entry through these channels, whereas hyperpolarization promotes Ca^{2+} entry. Cl$^-$ fluxes are likely to affect the membrane potential. We monitored membrane potential by using the potential-sensitive fluorescent dye, DiBAC$_4$(3), which shows a decrease in fluorescence upon hyperpolarization from the resting potential, whereas depolarization gives an increase in fluorescence.[26] When platelets were stimulated with thrombin-plus-CRP, DiBAC$_4$(3) fluorescence initially decreased, indicating hyperpolarization, followed by a small, gradual increase in fluorescence (Figure 4g). When Cl$^-$ channels were blocked the early hyperpolarization was completely lost (Figures 4g and h), and an underlying depolarization was revealed. The same effect was seen in Cl$^-$-free medium (Figure 4h). These data indicate that the early hyperpolarization is dependent on Cl$^-$ entry through Cl$^-$ channels.

Discussion

Although Ano6 is critically involved in Ca^{2+}-dependent PS exposure,[3,27] how it does this is not known. Several groups have shown that Ano6 can act as a Cl$^-$ channel in a range of cells.[7,14] If Ano6 is acting as a Cl$^-$ channel in platelets, then
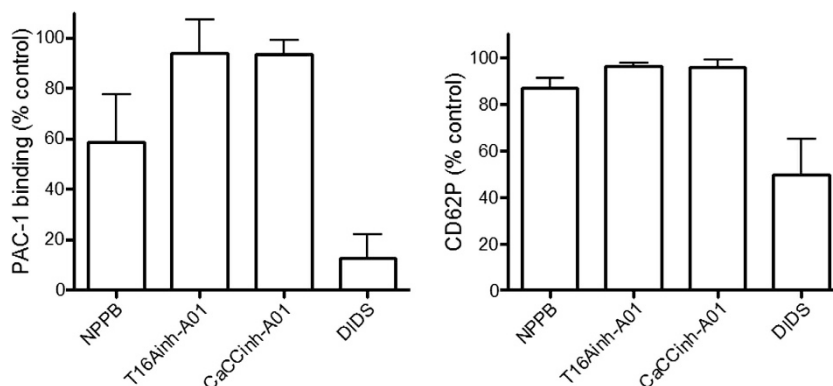
Figure 2 Effect of Cl^- channel blockers on integrin $\alpha_{IIb}\beta_3$ activation and α-granule secretion. Washed platelets were treated with the indicated Cl^- channel blockers (or DMSO) as control then stimulated with thrombin (1 U/ml) in the presence of FITC-conjugated PAC-1 antibody (left panel) or FITC-conjugated anti-CD62P antibody (right panel). Platelet staining was determined by flow cytometry and expressed as percentage of fluorescent signal in DMSO-treated, thrombin-stimulated platelets. ($n = 3$–4). Histograms depict mean $\pm$ S.E.M.
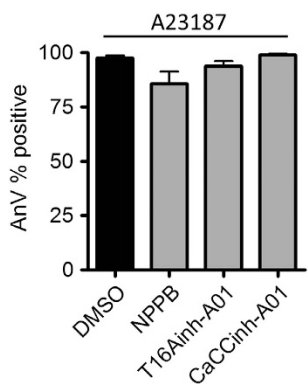
Figure 3 Chloride channels are not required for Ca^{2+}-dependent PS exposure. Washed platelets were stimulated with the Ca^{2+} ionophore, A23187 (10 μM), and annexin V (AnV) binding was monitored ($n = 4$). Histograms depict mean $\pm$ S.E.M.

Cl^- conductance might somehow regulate Ca^{2+}-dependent PS exposure. In this study we have tested whether Cl^- channels (whether comprising Ano6 or other proteins) can regulate platelet PS exposure. We conclude that although Cl^- channels are not required for platelets to expose PS in response to increased $[Ca^{2+}]_i$, they have a novel role in supporting platelet PS exposure by promoting Ca^{2+} entry, resulting in more rapid thrombin generation.

We propose that Cl^- entry through Cl^- channels is essential for platelet hyperpolarization. This maintains the driving force for Ca^{2+} entry through plasma membrane Ca^{2+} channels, leading to a sustained increase in $[Ca^{2+}]_i$. This is necessary to trigger full PS exposure and promote thrombin generation. In the absence of Cl^--dependent hyperpolarization, agonist stimulation leads to depolarization, probably due to Na^+ and Ca^{2+} entry. This reduces further Ca^{2+} entry, limits PS exposure and slows the rate of thrombin generation. In this way, Cl^- entry indirectly regulates PS exposure. The effect of preventing Cl^- entry is specific to Ca^{2+} entry as Ca^{2+} release from intracellular stores was not affected.

The molecular identities of platelet Cl^- channels involved are unknown. Ano6 remains a possibility if, as shown in some
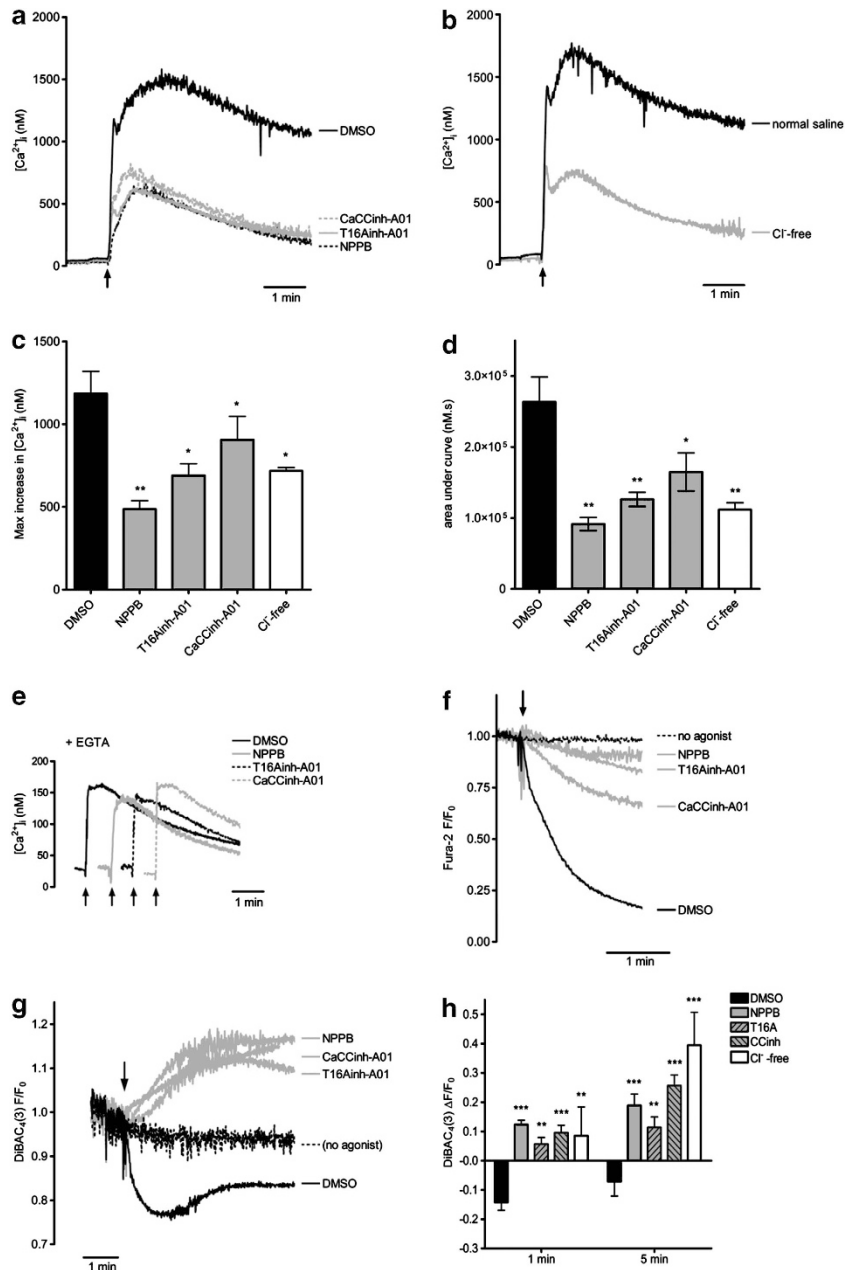
cells, Ano6 is a component of the VSOR Cl^- channel.[7] Conversely, it has been proposed that Ano6 is in fact a small conductance cation channel,[27] in which case the identity of the platelet Cl^- channel remains to be discovered.

Importantly, our data suggest that PS scrambling activity does not directly require Cl^- entry. Although our data show that Cl^- channels are important in platelet PS exposure, blocking Cl^- conductance does not completely abolish agonist-induced PS exposure and platelet-dependent thrombin generation and has no effect on A23187-induced PS exposure. A similar conclusion has recently been reached in lymphocytes by Kmit et al.,[14] who found that Cl^- channel blockers had no effect on ionomycin-induced PS exposure. This contrasts with the loss-of-function mutations in TMEM16F and absent Ca^{2+}-dependent PS exposure in Scott syndrome patients. Importantly, this implies that the role of Ano6 in Ca^{2+}-dependent PS exposure in platelets and lymphocytes is likely to be different to any putative function of Ano6 as a Cl^- channel.

Materials and Methods

Figure 4 Chloride channels mediate platelet membrane hyperpolarization and maintain Ca^{2+} entry. Intracellular Ca^{2+} concentration was monitored in fura-2-loaded human platelets (a–e). Platelets were stimulated with thrombin-plus-CRP as indicated by the arrows. Traces are representative of five independent experiments. The maximum increase in $[Ca^{2+}]i$ above basal and the area under the curve are quantified in (c) and (d), respectively ($n = 5$; *$P < 0.05$; **$P < 0.01$). (a, c) Platelets were treated with the indicated chloride channel blockers, or equivalent volume of DMSO as control, for 5 min before stimulation. (b, d) Platelets were suspended in normal NaCl-based buffer (–) or in Na-gluconate-based buffer (Cl^- free). (e) Platelets were stimulated in the absence of extracellular Ca^{2+} (1 mM EGTA added). (f) Mn^{2+} entry into fura-2-loaded platelets and quench of fura-2 fluorescence was used to monitor divalent cation entry. (g, h) Platelets were stimulated with thrombin and CRP in the presence of $DiBAC_4(3)$ in order to monitor membrane potential. Representative traces are shown in (g) and the mean changes in fluorescence ($\pm$ S.E.M.) at 1 and 5 min after stimulation are given in (h) ($n = 6$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

Thrombin generation. Thrombin generation was monitored in normalized PRP (1.5×10^8 platelets/ml). PRP was stimulated with CRP for 5 min. Thrombin generation was triggered with tissue factor/$CaCl_2$ cleavage of the substrate Z-Gly-Gly-Arg-AMC HCl measured by the thrombogram method in a Fluoroskan Ascent microplate reader at 37 °C. Thrombin concentrations were determined by using a Thrombin Calibrator (Stago) following correction for the inner filter effect and substrate consumption as described by Hemker *et al.*[15]

Flow cytometry. Annexin V-FITC was used to detect surface PS exposure. FITC-conjugated anti-CD62P antibody was used to monitor α-granule secretion and FITC-conjugated PAC-1 antibody was used to monitor $\alpha_{IIb}\beta_3$ activation. Platelets (5×10^7/ml) were stimulated in the presence of $CaCl_2$ (1 mM) for 10 min then diluted in Tyrode's-HEPES buffer containing 2 mM $CaCl_2$ and analysed immediately. Fluorescence was detected by flow cytometry, with platelets gated by their forward and side scatter profile Analysis of 20 000 platelets was performed

using a Becton Dickinson FACSCalibur (Oxford, UK). Data were analysed using WinMDI version 2.8 (The Scripps Research Institute, San Diego, CA, USA).

Calcium measurements. Fura-2 fluorescence from magnetically stirred cuvettes was monitored at 37°C using a Hitachi F-4500 (Hitachi High-Technologies, Maidenhead, UK) spectrofluorimeter with fluorescence excitation made at 340 and 380 nm, and emission at 510 nm. The ratio of emissions (F_{340}:F_{380}) was calibrated in terms of $[Ca^{2+}]_i$.[28] Calibrations were also performed in the presence of Cl^- channel blockers to account for any effects on fura-2 fluorescence.

Mn^{2+} entry. Fura-2 fluorescence was excited at the isosbestic wavelength (359 nm) and emission detected at 510 nm. Autofluorescence was subtracted and fluorescence was expressed relative to the initial fluorescence (F/F_0). Mn^{2+} entry was detected as a quench of fura-2 fluorescence.

Membrane potential measurement. Platelet membrane potential was monitored with the potential-sensitive fluorescent dye, DiBAC$_4$(3). Platelets were incubated with DiBAC$_4$(3) (100 nM; 30 min) prior to stimulation. Fluorescence was excited at 490 nm and emission detected at 590 nm. Fluorescence was expressed relative to the initial value (F/F_0).

Data analyses. Mean data are presented with standard errors; the number of replicates of each experiment, representing independent experiments from platelet preparations from different donors is reported in the figure legends. Data were analysed by one-way ANOVA, with Dunnett's test for multiple comparisons. $P < 0.05$ was considered statistically significant.

Conflict of Interest

The authors declare no conflict of interest.

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