

## SHORT COMMUNICATION



# Extracellular chloride is required for efficient platelet aggregation

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## Abstract

Anion channels perform a diverse range of functions and have been implicated in ATP release, volume regulation, and phosphatidylserine exposure. Platelets have been shown to express several anion channels but their function is incompletely understood. Due to a paucity of specific pharmacological blockers, we investigated the effect of extracellular chloride substitution on platelet activation using aggregometry and flow cytometry. In the absence of extracellular chloride, we observed a modest reduction of the maximum aggregation response to thrombin or collagen-related peptide. However, the rate of aggregation was substantially reduced in a manner that was dependent on the extracellular chloride concentration and aggregation in the absence of chloride was noticeably biphasic, indicative of impaired secondary signaling. This was further investigated by targeting secondary agonists with aspirin and apyrase or by blockade of the ADP receptor P2Y<sub>12</sub>. Under these conditions, the rates of aggregation were comparable to those recorded in the absence of extracellular chloride. Finally, we assessed platelet granule release by flow cytometry and report a chloride-dependent element of alpha, but not dense, granule secretion. Taken together these data support a role for anion channels in the efficient induction of platelet activation, likely via enhancement of secondary signaling pathways.

## Keywords

ADP, aggregation, chloride, ion channels  
platelets, thrombin

## History

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## Introduction

In contrast to the role of cations (i.e., Ca<sup>2+</sup>, K<sup>+</sup>, and Zn<sup>2+</sup>) [1–3], the contribution(s) of anions to platelet activation remains unclear. Anion channels perform diverse functions including regulatory volume decrease [4], phosphatidylserine exposure [5], and ATP release [6]. Early patch clamp recordings demonstrated functional expression of Ca<sup>2+</sup>-activated Cl<sup>-</sup> channels in platelets [7, 8] and a megakaryocyte-like DAMI cell line [9]. Proteomic [10] and transcriptomic [11] analyses have since identified numerous anion channels that may be expressed by platelets. Of these, functional expressions of CLIC1 (Intracellular Cl<sup>-</sup> channel-1) [12], TMEM16F [13], and pannexin-1 [14] have been confirmed. Indicative of a hemostatic role for anion channels, CLIC1- and TMEM16F-deficient mice have associated platelet-related bleeding phenotypes [12, 13]. Additionally, pannexin-1 channels have been shown to amplify platelet activation responses to threshold agonist concentrations [14]. These ATP-permeable channels are associated with inflammatory conditions and may contribute to atherosclerosis [15].

Given the lack of specific anion channel blockers, we focus on the effect of extracellular Cl<sup>-</sup> ([Cl<sup>-</sup>]<sub>o</sub>) substitution on platelet activation. Our experiments highlight a role for anion channels in modulating the rate of platelet aggregation.

## Methods

### Materials

Aspirin, apyrase, and thrombin were from Sigma (Poole, UK). AR-C66096 was from Tocris Bioscience (Bristol, UK). Cross-linked collagen-related peptide (CRP-XL) was prepared as described previously [16] and supplied by R. Fardale (Cambridge, UK). Unless indicated, all other reagents were from Sigma.

### Washed platelet preparation

This study was approved by the local Ethics Committee at Anglia Ruskin University. Human blood was collected from healthy volunteers following informed consent in accordance with the Declaration of Helsinki. Blood was collected into 11 mM sodium citrate and washed platelets were prepared as described previously [17]. Platelets were resuspended in a nominally calcium-free buffer containing (in mM) 145 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 10 glucose, 10 HEPES, titrated to pH 7.35 with NaOH. Where indicated, [Cl<sup>-</sup>]<sub>o</sub> was substituted by equimolar gluconate.

### Aggregometry

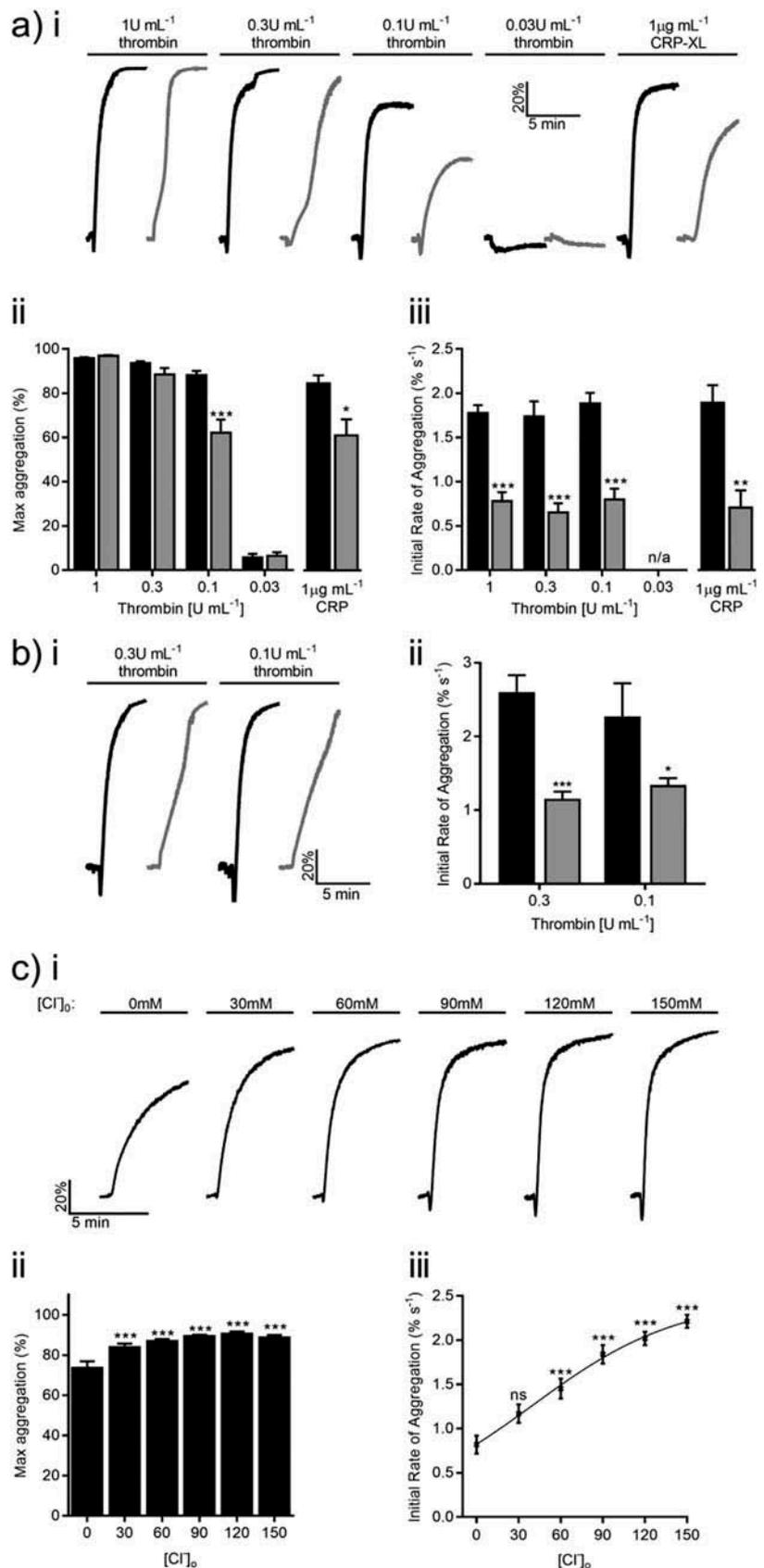
Platelet aggregation was monitored as described previously using an AggRam aggregometer (Helena Biosciences,

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Figure 1. Extracellular chloride is required for efficient platelet activation. (a) Washed platelets were stimulated by increasing concentrations of thrombin or  $1 \mu\text{g mL}^{-1}$  CRP-XL in the presence of 151 mM (black) or 1 mM (gray)  $[\text{Cl}^-]_o$ . Representative aggregation traces are shown for each condition (ai). Maximum aggregation (a(ii)) and the initial rate of aggregation (a(iii)) were calculated for each condition. (b) In the presence of 2 mM  $[\text{Ca}^{2+}]_o$ ,  $[\text{Cl}^-]_o$  substitution continued to reduce the initial rate of thrombin-evoked aggregation. (c) The sensitivity of thrombin-evoked ( $0.1 \text{ U mL}^{-1}$ ) aggregation to  $\text{Cl}^-$  was assessed by increasing  $[\text{Cl}^-]_o$  from 1 to 151 mM in 30 mM increments. Representative traces (ci), maximum (cii) and initial rate of thrombin-induced ( $0.1 \text{ U mL}^{-1}$ ) aggregation (ciii) for each  $[\text{Cl}^-]_o$  are shown. The rate of aggregation increased across the concentration range with an  $\text{EC}_{50} = 46.5 \pm 23.3 \text{ mM}$ . Data are representative of a minimum of four independent experiments. Thrombin and CRP-XL data sets were analyzed by two-way ANOVA and Student's *t* test, respectively.

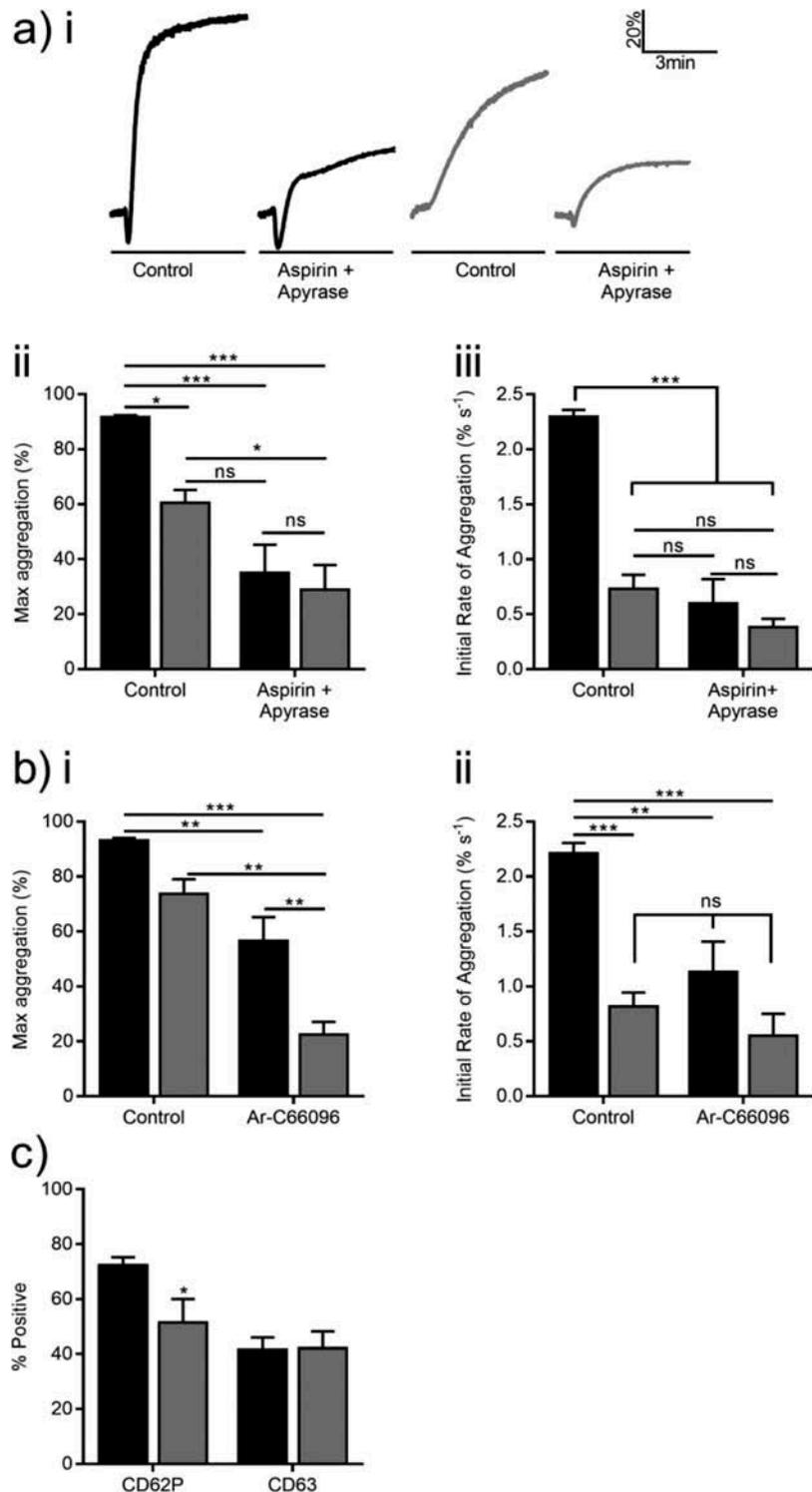


Gateshead, UK) [17]. In the experiments of Figure 1c, aliquots of 151 and 1 mM  $[\text{Cl}^-]_o$ -containing platelet suspensions were mixed 5 min prior to agonist addition. Platelets were preincubated with each drug(s) for 5 min at 37°C.

#### Granule release

Thrombin-evoked alpha and dense granule release was assessed by flow cytometry using fluorescently conjugated CD62P and CD63 antibodies (BD Biosciences, Oxford, UK),

**Figure 2.** Role for secondary signaling during  $\text{Cl}^-$ -dependent platelet aggregation. (a) Washed platelets were preincubated with  $100 \mu\text{M}$  aspirin (cyclooxygenase inhibitor) and  $5 \text{ U mL}^{-1}$  apyrase (ectonucleotidase) prior to performing aggregometry in the presence of  $151 \text{ mM}$  (black) or  $1 \text{ mM}$  (gray)  $[\text{Cl}^-]_o$ . Representative traces (ai), maximum (aii), and initial rate (aiii) of thrombin-induced ( $0.1 \text{ U mL}^{-1}$ ) aggregation are shown in the presence of vehicle control (0.1% ethanol) or aspirin plus apyrase. (b) Summary data for maximum (bi) and initial rate of thrombin-evoked ( $0.1 \text{ U mL}^{-1}$ ) platelet aggregation (bii) in the presence of vehicle control ( $\text{H}_2\text{O}$ ) or  $1 \mu\text{M}$  Ar-C66096 (P2Y<sub>12</sub> inhibitor). (c) Alpha (i) and dense (ii) granule release before (unstimulated) and after  $0.1 \text{ U mL}^{-1}$  thrombin stimulation was assessed by flow cytometry using fluorescently labeled CD62P and CD63 antibodies, respectively. Data are representative of a minimum of four independent experiments and data were analyzed by two-way ANOVA.



respectively. Antibody binding was monitored for 5 min using an Accuri C6 Flow cytometer (BD Biosciences) and the percentage of positive cells was calculated within FlowJo (V10.2, Oregon, USA).

#### Data analysis and statistics

Maximum aggregation (%) and the initial rate of aggregation ( $\% \text{ s}^{-1}$ ) were calculated in Excel (Microsoft, Redmond, Washington, USA), where rate was determined as the change in aggregation (%) in the first 30 s following shape change. Data were analyzed in GraphPad Prism by two-way ANOVA or Student's *t* test as indicated and are

representative of a minimum of four independent experiments. \*\*\*, \*\*, \*, and ns denote  $P < 0.001$ ,  $P < 0.01$ ,  $P < 0.05$ , and not significant, respectively.

#### Results

Platelet aggregation was assessed in the presence/absence of  $[\text{Cl}^-]_o$  in response to increasing thrombin concentrations (Figure 1a). Removal of  $[\text{Cl}^-]_o$  did not affect the maximum aggregation recorded across a 5-min time course in response to 0.03, 0.3, or  $1 \text{ U mL}^{-1}$  thrombin (Figure 1aii). A reduction from  $82.3 \pm 1.9\%$  to  $62.2 \pm 6.0\%$  ( $P < 0.001$ , Figure 1aiii) was observed at an

intermediate concentration ( $0.1 \text{ U mL}^{-1}$ ).  $\text{Cl}^-$  substitution affected the kinetics of platelet activation as the aggregation rate decreased by  $1.1 \pm 0.2\%$ ,  $1.1 \pm 0.2\%$ , and  $1.0 \pm 0.2\% \text{ s}^{-1}$  in response to stimulation by  $0.1$ ,  $0.3$ , and  $1 \text{ U mL}^{-1}$  thrombin, respectively ( $P < 0.01$ , Figure 1a<sub>iii</sub>). Similar effects were observed in the presence of  $2 \text{ mM Ca}^{2+}$  (Figure 1b), suggesting that this effect was not due to  $\text{Ca}^{2+}$  buffering. This observation was not exclusive to thrombin-evoked aggregation; removal of  $[\text{Cl}^-]_o$  reduced maximal CRP-XL-induced ( $1 \mu\text{g mL}^{-1}$ ) aggregation by  $23.5 \pm 3.9\%$  ( $P < 0.01$ , Figure 1a<sub>ii</sub>) and the rate decreased by  $1.2 \pm 0.1\% \text{ s}^{-1}$  ( $P < 0.001$ , Figure 1a<sub>iii</sub>). The sensitivity of thrombin-evoked ( $0.1 \text{ U mL}^{-1}$ ) aggregation to  $\text{Cl}^-$  was assessed by increasing  $[\text{Cl}^-]_o$  from  $1$  to  $151 \text{ mM}$  ( $30 \text{ mM}$  increments; Figure 1c). There was no change in maximum aggregation beyond  $31 \text{ mM}$   $[\text{Cl}^-]_o$  yet rates increased across the concentration range ( $\text{EC}_{50} = 46.5 \pm 23.3 \text{ mM}$ ).

Differences in aggregation rate may be due to reduced secondary signaling in the absence of  $[\text{Cl}^-]_o$ . Platelets were preincubated with  $100 \mu\text{M}$  aspirin and  $5 \text{ U mL}^{-1}$  apyrase to assess contributions by thromboxane  $\text{A}_2$  and extracellular nucleotides. These compounds reduced maximum aggregation in response to  $0.1 \text{ U mL}^{-1}$  thrombin by  $56.6 \pm 10.2\%$  ( $P < 0.001$ ) and  $31.6 \pm 10.2\%$  ( $P < 0.05$ , Figure 2a<sub>i</sub>, ii) in the presence of  $151$  and  $1 \text{ mM}$   $[\text{Cl}^-]_o$ , respectively. The rate of platelet aggregation following aspirin and apyrase treatment in the presence of  $151 \text{ mM}$  ( $0.6 \pm 0.2\% \text{ s}^{-1}$ ) or  $1 \text{ mM}$  ( $0.38 \pm 0.08\% \text{ s}^{-1}$ )  $[\text{Cl}^-]_o$  was comparable to that of  $1 \text{ mM}$   $[\text{Cl}^-]_o$  control ( $0.7 \pm 0.1\% \text{ s}^{-1}$ ;  $P > 0.05$ , Figure 2a<sub>iii</sub>).

P2Y<sub>12</sub>-mediated signaling is a major step during integrin  $\alpha_{\text{IIb}}\beta_3$  activation [18, 19] and reduced ADP availability may account for the observed aggregation defect.  $0.1 \text{ U mL}^{-1}$  thrombin-evoked aggregation in the presence of a P2Y<sub>12</sub> inhibitor ( $1 \mu\text{M}$  AR-C66096) decreased by  $36.7 \pm 8.2\%$  and  $51.2 \pm 7.4\%$  in the presence of  $151$  and  $1 \text{ mM}$   $[\text{Cl}^-]_o$  ( $P < 0.01$ , Figure 2b<sub>i</sub>), respectively. It is noteworthy that no differences between the aggregation rate of AR-C66096-treated platelets in  $151$  or  $1 \text{ mM}$   $[\text{Cl}^-]_o$  and the  $1 \text{ mM}$   $[\text{Cl}^-]_o$  control were observed ( $P > 0.05$ , Figure 2b<sub>ii</sub>), suggesting a role for P2Y<sub>12</sub> during  $\text{Cl}^-$ -dependent aggregation. Finally, we assessed thrombin-induced granule release by flow cytometry. Peak alpha granule release was reduced from  $72.4 \pm 2.9\%$  to  $59.9 \pm 1.6\%$  ( $P < 0.001$ , Figure 2c), while dense granule release was unaffected by  $\text{Cl}^-$  substitution ( $P > 0.05$ , Figure 2c).

## Conclusions

Here we demonstrate that  $[\text{Cl}^-]_o$  enhances the rate of platelet aggregation in a concentration-dependent manner (Figure 1). This effect was equivalent to blockade of secondary mediators and P2Y<sub>12</sub> inhibition (Figure 2). One possible explanation of these data is that  $[\text{Cl}^-]_o$  is required for efficient release of ATP and/or ADP from the platelet, but we failed to observe a change in dense granule secretion (Figure 2c). Pannexin-1 has been shown to activate in response to elevation of intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) [20], facilitating cytosolic ATP release [14]. It has been suggested that ADP release may occur via a similar mechanism [21]. Given that release of alpha granule cargo (e.g., fibrinogen, thrombin, and  $\text{Zn}^{2+}$ ) is required for aggregation and is enhanced by P2Y<sub>12</sub> signaling [22–24], it is possible that  $[\text{Cl}^-]_o$  enhances platelet activation by mediating efficient alpha granule secretion.

Reduction of  $[\text{Cl}^-]_o$  has been shown to substantially reduce thrombin-plus-CRP-XL-mediated elevation of  $[\text{Ca}^{2+}]_i$  in a similar manner to that of  $\text{Cl}^-$  channel blockers [25]. It has been suggested that  $\text{Cl}^-$  currents hyperpolarize the cell, increasing driving force for  $\text{Ca}^{2+}$  influx [9]. However, the platelet  $\text{Cl}^-$  equilibrium is  $\approx 35 \text{ mV}$  in the platelet [7], meaning activation of a  $\text{Cl}^-$  conductance would depolarize rather than hyperpolarize platelets. Reduced

$\text{Ca}^{2+}$  influx may be due to reduced secondary signaling, rather than altered membrane potential.

We have focused on the contribution of  $[\text{Cl}^-]_o$  by way of ionic substitution experiments because of the paucity of specific pharmacological tools to study anion channels. This may also explain why anion channels have previously received much less attention than cation channels. It is worth noting that anion channels have been associated with cystic fibrosis, bleeding phenotypes, and inflammatory conditions [12, 13, 15, 26, 27] and may represent valuable therapeutic targets, as demonstrated by clinical use of CFTR modulators [28]. Further work will be required to investigate the contribution(s) by the cohort of platelet anion channels during platelet activation.

## Declaration of interest

The authors report no declarations of interest.

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