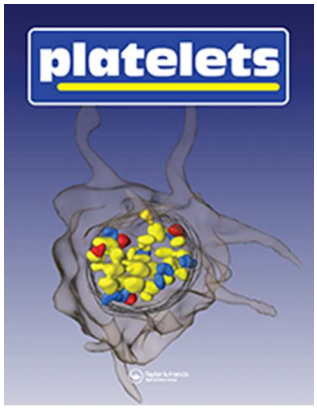

Extracellular Chloride is Required for Efficient Platelet Aggregation

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Short Title: Chloride Channels and Platelet Activation

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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 however 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 signalling. This was further investigated by targeting secondary agonists with aspirin and apyrase or by blockade of the ADP receptor P2Y12. 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 signalling pathways.

Introduction

In contrast to the role of cations (i.e. Ca^{2+}, K^{+} and Zn^{2+}) [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^{2+}-activated Cl^{-} 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 expression of CLIC1 (Intracellular Cl^{-} channel-1) [12], TMEM16F [13] and pannexin-1 [14] have been confirmed. Indicative of a haemostatic 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^{-} ($[Cl^{-}]_o$) substitution on platelet activation. These 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 biosciences (Bristol, UK). Cross-linked collagen-related peptide (CRP-XL) was prepared as described previously [16] and supplied by R. Farndale (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 11mM 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 $MgCl_2$, 10 glucose, 10 HEPES, titrated to pH 7.35 with NaOH. Where indicated, $[Cl^-]_o$ was substituted by equimolar gluconate.

Aggregometry: Platelet aggregation was monitored as described previously using an AggRam aggregometer (Helena Biosciences, Gateshead, UK) [17]. In the experiments of Fig. 1~~b~~c, aliquots of 151mM and 1mM $[Cl^-]_o$-containing platelet suspensions were mixed 5min prior to agonist addition. Platelets were pre-incubated with each drug(s) for 5min 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), respectively. Antibody-binding was monitored for 5min using an Accuri C6 Flow cytometer (BD Biosciences) and the percentage positive cells was calculated within FlowJo (V10.2, Oregon, USA).

Data Analysis and Statistics: Maximum aggregation (%) and the initial rate of aggregation (% s^{-1}) were calculated in Excel, where rate was determined as the change in aggregation (%) in

the first 30s following shape change. Data were analysed 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 *P>0.05*, respectively.

Results

Platelet aggregation was assessed in the presence/absence of $[Cl^-]_o$ in response to increasing thrombin concentrations (Fig. 1a). Removal of $[Cl^-]_o$ did not affect the maximum aggregation recorded across a 5min time course in response to 0.03, 0.3 or 1U mL^{-1} thrombin (Fig. 1aii). A reduction from 82.3±1.9% to 62.2±6.0% (*P<0.001*, Fig. 1aii) was observed at an intermediate concentration (0.1U mL^{-1}). Cl^- substitution affected the kinetics of platelet activation as the aggregation rate decreased by 1.1±0.2, 1.1±0.2 and 1.0±0.2% s^{-1} in response to stimulation by 0.1, 0.3 and 1U mL^{-1} thrombin, respectively (*P<0.01*, Fig. 1aiii). ~~A s~~Similar effects ~~were as~~ observed in the presence of ~~extracellular~~ 2mM Ca^{2+} (~~data not shown~~Fig. 1b), suggesting that this effect was not due to Ca^{2+} buffering. This ~~effect~~observation was not exclusive to thrombin-evoked aggregation, removal of $[Cl^-]_o$ ~~also~~ reduced maximal CRP-XL-induced (1µg mL^{-1}) aggregation by 23.5±3.9% (*P<0.01*, Fig. 1aii) and the rate decreased by 1.2±0.1% s^{-1} (*P<0.001*, Fig. 1aiii). The sensitivity of thrombin-evoked (0.1U mL^{-1}) aggregation to Cl^- was assessed by increasing $[Cl^-]_o$ from 1 to 151mM (30mM increments; Fig. ~~1b~~1c). There was no change in maximum aggregation beyond 31mM $[Cl^-]_o$ yet rates increased across the concentration range ~~with an~~(EC_{50}= 46.5±23.3mM).

Differences in aggregation rate may be due to reduced secondary signalling in the absence of $[Cl^-]_o$. Platelets were pre-incubated with 100µM aspirin and 5U mL^{-1} apyrase to assess contributions by thromboxane A_2 and extracellular nucleotides. These compounds reduced maximum aggregation in response to 0.1U mL^{-1} thrombin by 56.6±10.2% (*P<0.001*) and 31.6±10.2% (*P<0.*05, Fig. 2ai, ii) in the presence of 151mM and 1mM $[Cl^-]_o$, respectively. The rate of platelet aggregation following aspirin and apyrase treatment in the presence of

151mM (0.6±0.2% s^{-1}) or 1mM (0.38±0.08% s^{-1}) $[Cl^-]_o$- was comparable to that of 1mM $[Cl^-]_o$ control (0.7±0.1% s^{-1}; *P>0.05*, Fig. 2aiii).

P2Y12-mediated signalling is a major step during integrin $\alpha_{IIb}\beta3$ activation [18,19] and reduced ADP availability may account for the observed aggregation defect. 0.1U mL^{-1} thrombin-evoked aggregation in the presence of a P2Y12 inhibitor (1μM AR-C66096) decreased by 36.7±8.2% and 51.2±7.4% in the presence of 151mM and 1mM $[Cl^-]_o$ (*P<0.01*, Fig. 2bi), respectively. It is noteworthy that no differences between the aggregation rate of AR-C66096-treated platelets in 151 or 1mM $[Cl^-]_o$ and the 1mM $[Cl^-]_o$ control were observed (*P>0.05*, Fig. 2bii), suggesting a role for P2Y12 during Cl^--dependent aggregation. Finally, we assessed thrombin-induced granule release by flow cytometry. Peak alpha granule release was reduced from 72.4±2.9% to 59.9±1.6% (*P<0.001*, Fig. 2c), whilst dense granule release was unaffected by Cl^- substitution (*P>0.05*, Fig. 2c).

Conclusions

Here we demonstrate that $[Cl^-]_o$ enhances the rate of platelet aggregation in a concentration-dependent manner (Fig. 1). This effect was equivalent to blockade of secondary mediators and P2Y12 inhibition (Fig. 2). One possible explanation of these data is that $[Cl^-]_o$ is required for efficient release of ATP and/or ADP from the platelet, but we failed to observe a change in dense granule secretion (Fig. 2c). Pannexin-1 has been shown to activate in response to elevation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) [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 Zn^{2+}) is required for aggregation and is enhanced by P2Y12 signalling [22-24], it is possible that $[Cl^-]_o$ enhances platelet activation by mediating efficient alpha granule secretion.

Reduction of $[Cl^-]_o$ has been shown to substantially reduce thrombin-plus-CRP-XL-mediated elevation of $[Ca^{2+}]_i$ in a similar manner to that of Cl^- channel blockers [25]. It has been suggested that Cl^- currents hyperpolarise the cell, increasing driving force for Ca^{2+} influx [9]. However, the platelet Cl^- equilibrium is ≈35mV in the platelet [7], meaning activation of a Cl^- conductance would depolarise rather than hyperpolarise platelets. Reduced Ca^{2+} influx may be due to reduced secondary signalling, rather than altered membrane potential.

We have focused on the contribution of $[Cl^-]_o$ 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.

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Declaration of Interest

The authors report no declarations of interest.

References

1. Varga-Szabo D, Braun A, Nieswandt B. Calcium signaling in platelets. Journal of Thrombosis and Haemostasis. 2009;7(7):1057–1066.

2. McCloskey C, Jones S, Amisten S, Snowden RT, Kaczmarek LK, Erlinge D, Goodall AH, Forsythe ID, Mahaut-Smith MP. Kv1.3 is the exclusive voltage-gated K^+ channel of platelets and megakaryocytes: roles in membrane potential, Ca^{2+} signalling and platelet count. The Journal of Physiology. 2010;588(Pt 9):1399–1406.

3. Taylor KA, Pugh N. The contribution of zinc to platelet behaviour during haemostasis and thrombosis. Metallomics: Integrated Biometal Science. 2016;8(2):144–155.

4. Livne A, Grinstein S, Rothstein A. Volume-regulating behavior of human platelets. Journal of Cellular Physiology. 1987;131(3):354–363.

5. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. Nature. 2010;468(7325):834–838.

6. Bao L, Locovei S, Dahl G. Pannexin membrane channels are mechanosensitive conduits for ATP. FEBS letters. 2004;572(1–3):65–68.

7. Mahaut-Smith MP. Chloride channels in human platelets: evidence for activation by internal calcium. The Journal of Membrane Biology. 1990;118(1):69–75.

8. MacKenzie AB, Mahaut-Smith MP. Chloride channels in excised membrane patches from human platelets: effect of intracellular calcium. Biochimica Et Biophysica Acta. 1996;1278(1):131–136.

9. Sullivan R, Kunze DL, Kroll MH. Thrombin receptors activate potassium and chloride channels. Blood. 1996;87(2):648–656.

10. Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. Blood. 2012;120(15):e73-82.

11. Wright JR, Amisten S, Goodall AH, Mahaut-Smith MP. Transcriptomic analysis of the ion channelome of human platelets and megakaryocytic cell lines. Thrombosis and Haemostasis. 2016;116(2):272–284.

12. Qiu MR, Jiang L, Matthaei KI, Schoenwaelder SM, Kuffner T, Mangin P, Joseph JE, Low J, Connor D, Valenzuela SM, et al. Generation and characterization of mice with null mutation of the chloride intracellular channel 1 gene. Genesis (New York, N.Y.: 2000). 2010;48(2):127–136.

13. Yang H, Kim A, David T, Palmer D, Jin T, Tien J, Huang F, Cheng T, Coughlin SR, Jan YN, et al. TMEM16F forms a Ca^{2+}-activated cation channel required for lipid scrambling in platelets during blood coagulation. Cell. 2012;151(1):111–122.

14. Taylor KA, Wright JR, Vial C, Evans RJ, Mahaut-Smith MP. Amplification of human platelet activation by surface pannexin-1 channels. Journal of thrombosis and haemostasis: JTH. 2014;12(6):987–998.

15. Velasquez S, Eugenin EA. Role of Pannexin-1 hemichannels and purinergic receptors in the pathogenesis of human diseases. Frontiers in Physiology. 2014;5:96.

16. Asselin J, Gibbins JM, Achison M, Lee YH, Morton LF, Farndale RW, Barnes MJ, Watson SP. A collagen-like peptide stimulates tyrosine phosphorylation of syk and phospholipase C gamma2 in platelets independent of the integrin alpha2beta1. Blood. 1997;89(4):1235–1242.

17. Watson BR, White NA, Taylor KA, Howes J-M, Malcor J-DM, Bihan D, Sage SO, Farndale RW, Pugh N. Zinc is a transmembrane agonist that induces platelet activation in a tyrosine phosphorylation-dependent manner. Metallomics: Integrated Biometal Science. 2016;8(1):91–100.

18. Foster CJ, Prosser DM, Agans JM, Zhai Y, Smith MD, Lachowicz JE, Zhang FL, Gustafson E, Monsma FJ, Wiekowski MT, et al. Molecular identification and characterization of the platelet ADP receptor targeted by thienopyridine antithrombotic drugs. The Journal of Clinical Investigation. 2001;107(12):1591–1598.

19. Offermanns S. Activation of platelet function through G protein-coupled receptors. Circulation Research. 2006;99(12):1293–1304.

20. Locovei S, Wang J, Dahl G. Activation of pannexin 1 channels by ATP through P2Y receptors and by cytoplasmic calcium. FEBS letters. 2006;580(1):239–244.

21. Taylor KA, Wright JR, Mahaut-Smith MP. Regulation of Pannexin-1 channel activity. Biochemical Society Transactions. 2015;43(3):502–507.

22. Marx G, Korner G, Mou X, Gorodetsky R. Packaging zinc, fibrinogen, and factor XIII in platelet alpha-granules. Journal of Cellular Physiology. 1993;156(3):437–442.

23. Deppermann C, Cherpokova D, Nurden P, Schulz J-N, Thielmann I, Kraft P, Vögtle T, Kleinschnitz C, Dütting S, Krohne G, et al. Gray platelet syndrome and defective thrombo-inflammation in Nbeal2-deficient mice. The Journal of Clinical Investigation. 2013;123(8):3331–3342.

24. Harper MT, van den Bosch MT, Hers I, Poole AW. Platelet dense granule secretion defects may obscure α-granule secretion mechanisms: evidence from Munc13-4-deficient platelets. Blood. 2015;125(19):3034–3036.

25. Harper MT, Poole AW. Chloride channels are necessary for full platelet phosphatidylserine exposure and procoagulant activity. Cell Death & Disease. 2013;4:e969.

26. Castoldi E, Collins PW, Williamson PL, Bevers EM. Compound heterozygosity for 2 novel TMEM16F mutations in a patient with Scott syndrome. Blood. 2011;117(16):4399–4400.

27. Verkman AS, Galietta LJV. Chloride channels as drug targets. Nature reviews. Drug discovery. 2009;8(2):153–171.

28. Quon BS, Rowe SM. New and emerging targeted therapies for cystic fibrosis. BMJ (Clinical research ed.). 2016;352:i859.

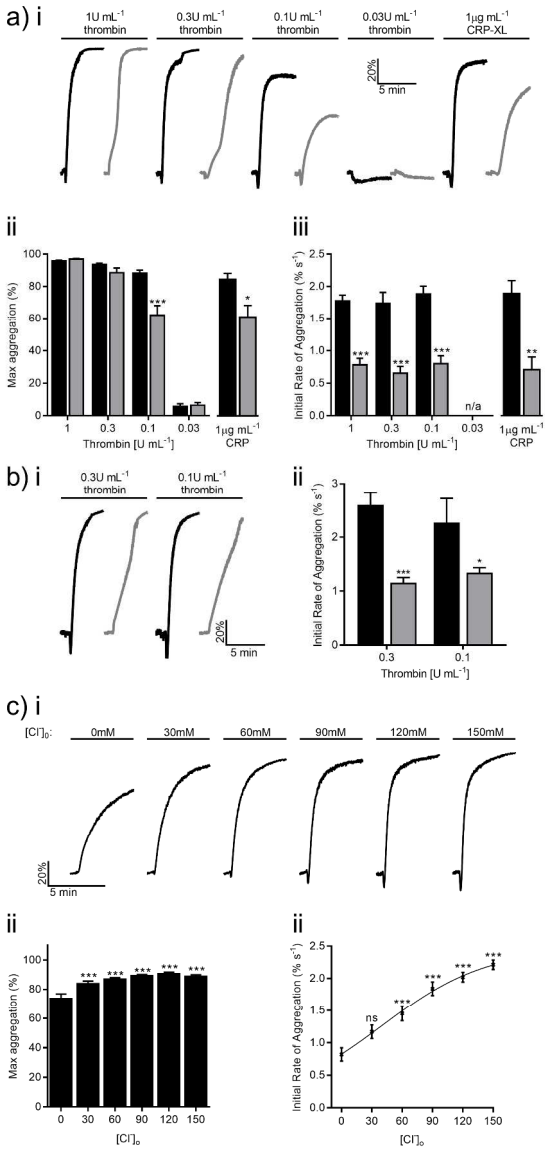

Figure 1: Extracellular chloride is required for efficient platelet activation. (a) Washed platelets were stimulated by increasing concentrations of thrombin or 1µg mL^{-1} CRP-XL in the presence of 151mM (black) or 1mM (grey) $[Cl^-]_o$. Representative aggregation traces are shown for each condition (ai). Maximum aggregation (aii) and the initial rate of aggregation (aiii) were calculated for each condition. (b) Removal of $[Cl^-]_o$ continued to reduce the initial rate of thrombin-evoked aggregation in the presence of 2mM $CaCl_2$. (c) The sensitivity of thrombin-evoked (0.1U mL^{-1}) aggregation to Cl^- was assessed by increasing $[Cl^-]_o$ from 1 to 151mM in 30mM increments. Representative traces (ci), maximum (cii) and initial rate of thrombin-induced (0.1U mL^{-1}) aggregation (ciii) for each $[Cl^-]_o$ are shown. The rate of aggregation increased across the concentration range with an EC_{50}= 46.5±23.3mM. Data are representative of a minimum of four independent experiments. Thrombin and CRP-XL data sets were analysed by two-way ANOVA and Student's t test, respectively.

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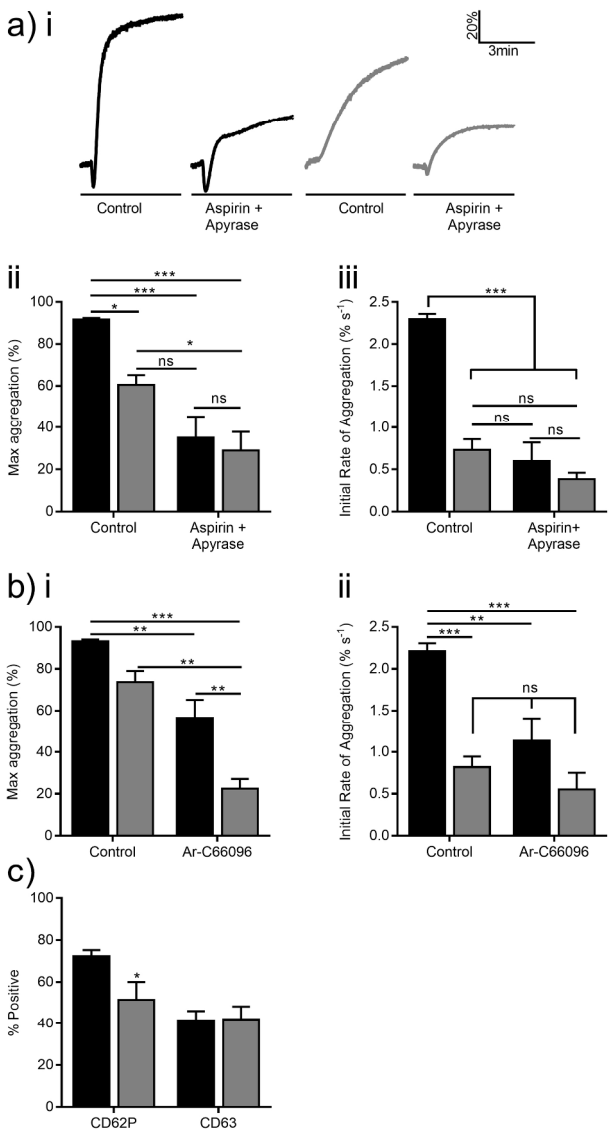

Figure 2: Role for secondary signalling during Cl^--dependent platelet aggregation. (a) Washed platelets were pre-incubated with 100μM aspirin (cyclooxygenase inhibitor) and 5U mL^{-1} apyrase (ectonucleotidase) prior to performing aggregometry in the presence of 151mM (black) or 1mM (grey) $[Cl^-]_o$. Representative traces (ai), maximum (aii) and initial rate (aiii) of thrombin-induced (0.1U 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 (0.1U mL^{-1}) platelet aggregation (bii) in the presence of vehicle control (H2O) or 1μM Ar-C66096 (P2Y12 inhibitor). (c) Alpha (i) and dense (ii) granule release before (Unstim.) and after 0.1U mL^{-1} thrombin stimulation was assessed by flow cytometry using fluorescently-labelled CD62P and CD63 antibodies, respectively. Data are representative of a minimum of four independent experiments and data were analysed by two-way ANOVA.

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