The Plasma Membrane Calcium ATPase (PMCA) modulates calcium homeostasis, intracellular signalling events and functionality in platelets

Running head: PMCA and platelet function


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Word count:  
Abstract: 217  
Manuscript: 3859
Summary

Background: The Plasma Membrane Calcium ATPase (PMCA) regulates localized signalling events in a variety of cell types although its functional role in platelets remains undefined.

Objectives: To investigate the role of PMCA in determining platelet $[\text{Ca}^{2+}]_i$, at rest and following agonist stimulation and define the corresponding effects upon different stages of platelet activation. Methods: $[\text{Ca}^{2+}]_i$ was continuously measured in Fura2 loaded platelets and in vitro and in vivo functional analyses performed in the presence of the PMCA inhibitor carboxy eosin (CE). Results: Concentrations of CE that selectively inhibited $\text{Ca}^{2+}$ extrusion through PMCA were established in human platelets. $[\text{Ca}^{2+}]_i$ was elevated by CE in resting platelets although collagen stimulated $\text{Ca}^{2+}$ release was reduced. Impaired $\text{Ca}^{2+}$ mobilisation upon agonist stimulation was accompanied by reduced dense granule secretion and impaired platelet aggregation. Platelet aggregation responses were also reduced in PMCA4$^{-/-}$ mice and in an in vivo mouse model of platelet thromboembolism. Conversely, inhibition of PMCA promoted the early and later stages of platelet activation observed as enhanced adhesion to fibrinogen, and accelerated clot retraction. Investigations into the signalling mechanisms underlying CE-mediated inhibition of platelet aggregation implicated cGMP-independent Vasodilator-Stimulated Phosphoprotein (VASP) phosphorylation. Conclusions: Disruption of PMCA activity perturbs platelet $\text{Ca}^{2+}$ homeostasis and function in a time-dependent manner demonstrating that PMCA differentially regulates $\text{Ca}^{2+}$-dependent signalling events and hence function throughout the platelet activation process.

Key Words: calcium, pharmacology, platelet, signalling, thrombosis
Introduction

An increase in intracellular calcium concentration ([Ca$^{2+}$]$_i$) and subsequent stimulation of localized Ca$^{2+}$-dependent signalling processes is central to platelet activation. Platelet Ca$^{2+}$ homeostasis is therefore tightly regulated to maintain low resting Ca$^{2+}$ levels that rise with spatiotemporal specificity upon activation [1].

The Plasma Membrane Calcium ATPase (PMCA) has been described as the predominant route of Ca$^{2+}$ extrusion across the platelet plasma membrane [2] although there is increasing evidence of a functional role of the Na$^+$/Ca$^{2+}$ exchanger. Na$^+$/Ca$^{2+}$ exchange has been shown to regulate basal Ca$^{2+}$ [3] and in reverse mode can increase [Ca$^{2+}$], and promote platelet aggregation [4, 5] by influencing cytoskeletal function [5] and dense granule secretion [6]. Thus, the distinct role of Ca$^{2+}$ extrusion through PMCA in regulating Ca$^{2+}$ homeostasis and function in platelets remains unclear.

PMCA isoforms are encoded by four independent genes. Alternative splicing of the primary constructs of these genes results in approximately 30 PMCA isoforms [7]. Platelets express PMCA4b and to a lesser extent PMCA1b [8]. Traditionally, PMCA has been thought to play a “housekeeping” role in controlling basal Ca$^{2+}$ levels [9] but more recently has been shown to act as a versatile signalling molecule [10], for example by forming interactions with PDZ-domain containing proteins such as neuronal nitric oxide synthase (nNOS) to produce functionally important effects including changes in contractile function in the heart [11].

In platelets, PMCA interacts with the actin cytoskeleton and localizes to the filopodal region during activation [12] suggesting roles that are temporally and spatially dependent. The roles of PMCA in regulating [Ca$^{2+}$], and function during the various stages of platelet activation are not known. In the present study, the role of PMCA in regulating platelet [Ca$^{2+}$], and function was investigated using the PMCA inhibitor carboxyeosin (CE). We established
concentrations of CE that inhibited $\text{Ca}^{2+}$ extrusion through PMCA in platelets and confirmed that CE acted selectively at PMCA. The consequences of PMCA inhibition on $\text{Ca}^{2+}$ homeostasis and platelet function were then determined and the validity of our functional observations confirmed with parallel experiments in PMCA4 knock-out (PMCA$^{4/-}$) mice. Mechanistic studies were initiated as a first step in elucidating the pathways linking PMCA activity with functionality in platelets.
Materials and methods

Materials

Materials were purchased as follows: \(^{3}\text{H}\) 5-HT and \(^{111}\text{In}\) Indium oxine (GE-Healthcare, Amersham, UK); collagen (Nycomed, Munich, Germany); carboxyeosin (CE) and p-nitrophenyl phosphate (Invitrogen, Paisley, UK); anti-VASP and anti-phospho-VASP (Ser239) (New England Biolabs, Hitchin, UK). All other materials were purchased from Sigma-Aldrich (Poole, UK). Tyrodes-HEPES buffer contained 134mM NaCl, 2.9mM KCl, 12mM NaHCO\(_3\), 0.34mM Na\(_2\)HPO\(_4\), 20mM HEPES, 5mM glucose, 1mM MgCl\(_2\), pH 7.3.

Platelet preparation

Blood was taken from aspirin-free human volunteers or terminally anaesthetized mice and washed platelets prepared by differential centrifugation as previously described [13, 14]. Informed consent from all blood donors was obtained and procedures were approved by the National Research Ethics Service.

Intracellular calcium measurements

Human platelets were loaded with Fura2 by incubation with 5 \(\mu\)M Fura2-AM and washed in modified Tyrodes-HEPES buffer before re-suspension to a density of approximately \(4 \times 10^8\) cells/ml. Platelets were incubated with CE for 30 min and \([\text{Ca}^{2+}]_i\) measurements taken using a LS50B fluorescence spectrometer (Perkin Elmer, UK) in collagen (5 \(\mu\)g/ml) or thapsigargin (1 \(\mu\)M) plus ionomycin (50 nM) stimulated or resting platelets. Unless otherwise stated, \([\text{Ca}^{2+}]_i\) analyses were conducted in \([\text{Ca}^{2+}]_i\)-free buffer in the presence of 100 \(\mu\)M EGTA.
Platelet aggregation

Platelets were incubated for 30 min with CE (10-40 µM) or DMSO (0.4%) prior to stimulation with collagen (5 µg/ml) or thrombin (0.1 U/ml) and aggregation measured at 37°C under stirring conditions in an optical aggregometer (Chrono-log Corporation, Havertown, PA, USA). Where sodium nitroprusside (SNP) and ODQ (1H-[1,2,4] Oxadiazolo[4,3-a] quinoxalin-1-one) were used, platelets were incubated with these compounds for 60 sec prior to stimulation. Mouse studies were conducted as above by isolating platelets from pooled groups of wild-type and PMCA4^{−/−} mice. Unless otherwise indicated all aggregation studies were conducted in modified Tyrodes-HEPES buffer containing 1 mM CaCl₂.

Dense granule secretion

Washed platelets pre-loaded with [³H]5-hydroxytryptamine ([³H]5-HT; 0.5 µCi/ml) were incubated for 30 min with CE (10-40 µM) or DMSO (0.4%) prior to stimulation with collagen (5 µg/ml) for 180 sec under stirring conditions. [³H]5-HT release was measured in supernatants by scintillation counting.

Platelet adhesion

96 well plates were coated with 100 µg/ml fibrinogen for 1 hr at room temperature and blocked with 1% BSA for 1 hr. Washed platelets treated with CE (10-40 µM) were incubated in wells for 1 hr at 37 °C, and wells washed 3 times with Tyrodes-Hepes buffer prior to
incubation with acid phosphatase buffer for 1 hr at 37 °C. 2 mM NaOH was then added to the wells and absorbance measured at 405 nm.

**Clot retraction**

PRP (200 µl) was incubated with DMSO (0.4%) or CE (20 or 40 µM) for 30 min before being transferred to a glass test tube containing Tyrodes-HEPEs buffer (750 µl). Erythrocytes were added to enhance colour for photographic purposes before thrombin (2.5 U/ml) was added and a glass rod inserted to initiate clot formation. After 2 hr at room temperature the clots were photographed and weighed.

**Immunoblotting**

Proteins were separated using sodium dodecyl sulphate-polyacrylamine electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked using 5% (w/v) milk and incubated with anti-phospho-VASP (Ser239) or anti-VASP (1:1000 in TBST, 2% BSA) overnight at 4 °C. Blots were washed and incubated with horseradish-peroxidase-conjugated secondary antibody (1:5000 in TBST, 2% BSA) prior to detection using an enhanced chemiluminescence detection system (ECL).

**Mice**

Male Balb/c mice (20-30 g) were purchased from Harlan (Bicester, UK). PMCA4<sup>Δ/-</sup> mice were bred under a heterozygous breeding programme and genotyped according to published protocols [15]. All protocols involving the use of animals were licensed by the UK Home
Office, approved by the Ethical Review Panel at Imperial College London and refined in association with the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) [14].

In vivo platelet thromboembolism

A mouse model of platelet thromboembolism developed in our laboratory was employed [14]. Blood was collected from terminally anaesthetized donor mice by cardiac puncture and platelets were isolated and incubated with 1.8 MBq [111]Indium oxine for 10 min. Platelets were then re-suspended in modified Tyrodes-HEPES buffer in the presence of CE (20 µM or 40 µM) or DMSO (0.4%). Anaesthetized mice were infused with radiolabelled platelets prior to administration of collagen (25-75 µg/kg i.v.). Platelet thromboembolic responses were measured in the pulmonary region as changes in platelet-associated counts using a Single Point Extended Area Ratio (SPEAR) detector (eV Products, Saxonburg, PA) and recorded using custom software (Mumed systems, London, UK) as previously described [14].

Data analysis and statistics

All data are expressed as mean ± S.E.M. Where statistical comparisons were made a Students’ t-test or one-way analysis of variance followed by a multiple comparison test was used to compare mean values and a p value < 0.05 denoted statistical significance.
Results

PMCA regulates \([Ca^{2+}]_i\) in resting and stimulated platelets.

In control platelets loaded with Fura2 and monitored in \(Ca^{2+}\)-free media in the presence of EGTA to assess release of stored \(Ca^{2+}\), thapsigargin plus ionomycin (Tg + Iono) induced a transient increase in \([Ca^{2+}]_i\) that decayed towards baseline due to \(Ca^{2+}\) release from internal stores and extrusion through PMCA as previously reported [2] (Fig 1A). Pre-treatment of platelets with 10-40 \(\mu\)M CE dose-dependently inhibited \(Ca^{2+}\) extrusion through PMCA shown as an impaired \(Ca^{2+}\) decay following Tg + Iono (Fig 1A). There was no evidence of an effect of CE on store loading since \(Ca^{2+}\) release (measured as peak minus basal) was unaffected by this treatment (Fig 1A and Supplementary Fig 1).

CE also dose-dependently increased \([Ca^{2+}]_i\) in resting platelets (Fig 1B), indicating a role for PMCA in maintaining basal \(Ca^{2+}\) levels.

The peak \([Ca^{2+}]_i\) following collagen stimulation was reduced in CE pre-treated platelets indicating reduced collagen mediated \(Ca^{2+}\) release (Fig 1C). Time-course experiments revealed that \([Ca^{2+}]_i\) in control platelets had returned to basal levels 180s after stimulation, whereas in platelets incubated with CE, \([Ca^{2+}]_i\) remained elevated beyond this time (Fig 1D).

Thus, CE reduces the amplitude of the initial \(Ca^{2+}\) transient following collagen stimulation but leads to an elevated response at later time points.

Validation of carboxyeosin as a PMCA inhibitor

Since the pharmacologically active, free acid form of CE is eosin, which is fluorescently active, we conducted a series of experiments to determine if eosin could dose-dependently increase Fura2 emission independent of \([Ca^{2+}]\) and thus invalidate data presented in Fig 1. A
broad range of eosin concentrations (0.1 – 50 µM) caused an apparent suppression of 340/380 emission that was neither significant nor dose-dependent (see Supplementary Fig 2A).

Although, excess CE was removed from platelets prior to analysis, we could not guarantee its absence from our preparations and so we additionally demonstrated that CE (0.1 – 50 µM) did not affect Fura2 340/380 emission (see Supplementary Fig 2B). Thus, we conclude that although the observed increases in Fura2 emission in resting platelets containing eosin may have been suppressed (i.e. underestimated) and quantitative studies could not be performed, our finding of a dose-dependent increase in Fura2 emission with CE and our conclusion that PMCA regulates basal [Ca\(^{2+}\)]\(_i\) is valid.

We were also able to observe a similar increase in basal Ca\(^{2+}\) to that observed with CE by incubating platelets with the nonselective cation channel antagonist lanthanum (1 mM) (Supplementary Fig 3) confirming a comparable increase in Ca\(^{2+}\) following inhibition of Ca\(^{2+}\) extrusion through an independent mechanism and in the absence of potentially confounding fluorescence issues. In addition, the effect of CE on Ca\(^{2+}\) homeostasis did not occur in platelets pretreated with lanthanum, indicating that the effect of CE on Ca\(^{2+}\) homeostasis was exerted at the plasma membrane rather than at a location independent of PMCA (Supplementary Fig 3).

Since CE has been shown to inhibit Na\(^+\)/K\(^+\)-ATPase pumps [16] as well as PMCA we investigated the effects of the Na\(^+\)/K\(^+\)-ATPase inhibitor ouabain on platelet aggregation. Concentrations of ouabain previously shown to inhibit Na\(^+\)/K\(^+\)-ATPases in platelets (1-10 µM [17, 18]) and excess concentrations (200 µM) had no effect on collagen or thrombin induced platelet aggregation (see Supplementary Figs 4A and B respectively).

\textit{PMCA positively regulates platelet aggregation and secretion}
CE dose-dependently inhibited collagen (Fig 2A) and thrombin (Supplementary Fig 5A) mediated human platelet aggregation indicating a positive regulatory role of PMCA in these processes. CE also inhibited collagen evoked 5-HT secretion (Fig 2B) and inhibited collagen evoked aggregation of mouse platelets (Supplementary Fig 5B). Collagen induced platelet aggregation responses were also significantly reduced in platelets from PMCA4−/− mice compared with those from wild-type controls (Figs 2C and D). Finally, the inhibitory effect of CE observed in platelets from wild-type mice did not occur in platelets from PMCA4−/− mice which aggregated normally in the presence of CE (Fig 2 E and F).

**PMCA regulates platelet function in vivo.**

CE dose-dependently impaired platelet aggregatory responses to collagen in vivo (Fig 3A). The maximal percentage increase in platelet-associated counts in response to collagen was significantly reduced by CE (Fig 3B) indicating reduced platelet responsiveness in vivo following PMCA inhibition.

**PMCA negatively regulates early and late stages of platelet activation.**

Although agonist induced Ca^{2+} release was suppressed by CE (Fig 1C), [Ca^{2+}], was elevated in resting platelets (Fig 1B) and in the later stages of activation (Fig 1D). To establish whether these temporal differences in [Ca^{2+}], impacted platelet function, we determined the consequences of PMCA inhibition on platelet adhesion and clot retraction to assess the early and later stages of platelet activation respectively.

In contrast to platelet aggregation and secretion, which were suppressed following PMCA inhibition (Fig 2), CE significantly enhanced platelet adhesion to fibrinogen (Fig 4A) and
dose-dependently reduced clot weight (Figs 4B-C) indicating enhanced clot retraction. Thus, PMCA positively regulates platelet aggregation but negatively regulates platelet adhesion and clot retraction.

**PMCA inhibition does not desensitize platelets.**

To assess whether pre-stimulation due to raised basal [Ca$^{2+}$]_i [19] was the mechanism underlying the inhibition of platelet aggregation observed with CE, platelet secretion and aggregation were measured in resting platelets incubated with CE. For contrast, secretion and aggregation were also determined in resting platelets incubated with the SERCA inhibitor thapsigargin which also leads to increased [Ca$^{2+}$]_i [20].

Thapsigargin dose-dependently increased dense granule secretion (Supplementary Fig 6A) and light transmission (Supplementary Fig 6B) in unstimulated platelets indicating pre-stimulation. When thapsigargin pre-treated platelets were subsequently stimulated with collagen (Supplementary Fig 6B) there was no difference in platelet aggregation compared to DMSO controls.

In contrast, there was no significant increase in dense granule secretion during incubation with CE at concentrations shown to inhibit aggregation responses (Supplementary Fig 6C). Similarly, light transmission in unstimulated platelets was not significantly increased by CE, with the exception of 40 µM where there was a small but significant increase of 5.3 ± 1.6% (Supplementary Fig 6D).

**CE enhances VASP phosphorylation independently of cGMP**
Western blots clearly demonstrate a dose-dependent increase in VASP Ser239 phosphorylation in platelets incubated with CE (Fig 5A), which were normalized to total VASP levels (Fig 5B).

The NO donor sodium nitroprusside (SNP, 1 µM) almost completely inhibited collagen mediated aggregation and this inhibition was reversed by the guanylyl cyclase inhibitor ODQ (10 µM) indicating effective inhibition by ODQ (Fig 5C). CE induced inhibition of aggregation, however, was not impaired by ODQ suggesting that the inhibition caused by CE, although mediated through VASP phosphorylation, was independent of cGMP.
Discussion

The ability of PMCA to regulate Ca\(^{2+}\)-dependent signalling events and hence function in a variety of cell types [10] led us to investigate its role in platelet Ca\(^{2+}\) homeostasis and relate this to the various stages of platelet activation. This is an essential step in elucidating the signalling and functional events regulated by PMCA in the platelet.

We initially determined concentrations of CE that effectively inhibited Ca\(^{2+}\) extrusion through PMCA by showing impaired extrusion under experimental conditions in which PMCA activity predominates [2]. We then used these effective concentrations of CE to ascertain the role of PMCA in regulating Ca\(^{2+}\) homeostasis and function in human platelets.

We had originally hypothesised that inhibition of PMCA would impair Ca\(^{2+}\) extrusion and elevate [Ca\(^{2+}\)], leading to platelet activation by sub-threshold concentrations of platelet agonists. Early experiments however demonstrated that while CE elevated basal [Ca\(^{2+}\)], demonstrating its role in the maintenance of low resting [Ca\(^{2+}\)], it inhibited agonist induced intracellular Ca\(^{2+}\) release indicating that PMCA somehow propagates the rise in [Ca\(^{2+}\)] following agonist stimulation. Further investigation revealed that reduced Ca\(^{2+}\) mobilisation following PMCA inhibition was accompanied by impaired dense granule secretion and aggregation, consistent with PMCA being a positive driving force behind these processes. Moreover, inhibition of PMCA resulted in platelets being unable to function normally in vivo confirming the relevance of our observations to physiological and pathological processes.

The validity of our data with CE relies on its specificity as an inhibitor of PMCA. CE crosses the plasma membrane and is then cleaved by intracellular esterases to yield eosin. Eosin interacts with amine groups in proteins and inhibits PMCA independent of the ATPase site [21, 22]. In terms of specificity, CE inhibits PMCA at the concentrations employed here without inhibiting SERCA or Na\(^+\)-Ca\(^{2+}\) exchange [21-23] and in line with this we were able
to demonstrate normal store loading in the presence of CE since there was no change in
thapsigargin plus ionomycin induced Ca\(^{2+}\) release calculated from data shown in Fig 1A as
peak response minus stable pre-stimulation values (see Supplementary Fig 1). CE has been
used to determine the function of PMCA in cardiomyocytes [23], sperm [15], embryonic
stem cells [24] and vascular endothelial cells [25]. Critical to our argument, the validity of
these functional observations has been confirmed by studies in PMCA4 knock-out and
transgenic lines [11, 15, 26, 27] suggesting a predominant action of CE at PMCA in a range
of cell types. CE does, however, inhibit Na\(^+/K^+\)-ATPase activity at micromolar
concentrations [16] and effects on transients during Ca\(^{2+}\) repletion [18] and procoagulant
effects [28] have been reported. We could find no published evidence of a role of this pump
in modulating platelet aggregation and demonstrated here that the Na\(^+/K^+\)-ATPase inhibitor
ouabain had no effect on platelet aggregation under our experimental conditions. Thus, the
effect of CE on platelet aggregation is unlikely to be mediated through inhibition of Na\(^+/K^+\)-
ATPase activity. Since the complete inhibitory profile of CE is unknown, we also conducted
experiments in PMCA4\(^{-/-}\) mice to determine whether a similar inhibition of platelet
aggregation was observed in the absence of confounding non-specific pharmacological
effects. Platelet aggregation was significantly reduced in PMCA4\(^{-/-}\) mice. The occurrence of
the same functional observation following both pharmacological inhibition of PMCA and
genetic ablation of PMCA4 provides strong evidence that the observed impairment of platelet
aggregation in both situations occurs as a result of loss of PMCA activity. This was further
evidenced by our data indicating that CE inhibited aggregation in WT but not PMCA4\(^{-/-}\) mice
(Fig 2E-F). These data also suggest that although CE inhibits all isoforms of PMCA, its
effects in platelets are mediated via inhibition of PMCA4 and that the PMCA4 isoform is a
key modulator of platelet function.
In contrast to the inhibitory effect of CE on platelet aggregation, platelet adhesion and clot retraction were enhanced. Increased platelet adhesion to fibrinogen and elevated basal Ca\(^{2+}\) prior to this response suggest that in the early stages of platelet activation PMCA acts to suppress both \([Ca^{2+}]_i\) and activation, consistent with the Ca\(^{2+}\) dependence of the fibrinogen receptor integrin α\(_{\text{IIb}}\)β\(_3\) which mediates adhesion. Similarly, enhanced clot retraction occurred against a background of prolonged elevations of \([Ca^{2+}]_i\) (Fig 1D). These elevations are consistent with the Ca\(^{2+}\) dependence of myosin activity which is essential for the clot retraction process. Thus PMCA acts to slow the rate at which clots retract presumably to ensure effective healing of underlying damaged tissue. Our clot retraction data are consistent with results published by Dean and colleagues [12] demonstrating that preventing translocation of PMCA to the filopodia of activated platelets enhanced clot retraction. Together these two data sets demonstrate the importance of both the locality and functionality of PMCA in regulating the clot retraction process.

Possible mechanisms by which PMCA inhibition could reduce platelet aggregation include partial activation and subsequent desensitization of platelets due to elevated \([Ca^{2+}]_i\), or coupling of PMCA with a Ca\(^{2+}\)-dependent negative regulator of platelet function. The former hypothesis was explored first. It has recently been reported that a mutation of stromal interaction molecule 1 (STIM1), a Ca\(^{2+}\) sensor situated in the endoplasmic reticulum, leads to elevated \([Ca^{2+}]_i\), in platelets, resulting in a pre-activation state and reduced responses to collagen [19]. We could not, however, find evidence of pre-activation since we were unable to detect secretion or aggregation during pre-incubation with CE. Furthermore, elevation of \([Ca^{2+}]_i\), through SERCA inhibition, despite stimulating dense granule secretion and partial aggregation, did not desensitize platelets to collagen. Thus, we confirmed that our methodologies for detecting platelet pre-activation against a background of elevated \([Ca^{2+}]_i\),
were effective and that elevating \([\text{Ca}^{2+}]\), in platelets did not \textit{per se} lead to inhibition of subsequent agonist induced aggregation.

Very little PMCA is associated with the platelet cytoskeleton under resting conditions but upon stimulation approximately 80% of the total PMCA is redistributed by the cytoskeleton and retains ATPase activity [29]. This redistribution potentially enables \text{Ca}^{2+} \text{and signalling molecules, potentially inhibitory signalling pathways, to be regulated locally. Such a role of PMCA in regulating inhibitory signalling pathways has already been demonstrated in cardiomyocytes [11] and vascular smooth muscle cells [26] where PMCA exerts its functional effects by regulating nNOS activity. Thus the application of CE in our study could inhibit platelet aggregation by raising \text{Ca}^{2+} in the vicinity of localized inhibitory signalling molecules such as those regulating VASP activity. VASP-Ser239 phosphorylation was shown to be increased by CE indicating that PMCA prevents the phosphorylation of VASP.

One of the major mechanisms of Ser239 VASP phosphorylation in platelets is increased cGMP production by nitric oxide (NO) and other mediators such as vWF [30]. In our study, the inhibition of platelet aggregation by CE occurred independently of cGMP. Although Ser239 is considered the preferred phosphorylation site for cGMP/PKG, the site has also been shown to be both PKG- and PKA- sensitive [31, 32], indicating that PMCA inhibition may stimulate alternative \text{Ca}^{2+}-dependent inhibitory pathways in platelets. As research continues into the role of PMCA, novel interactions between PMCA and signalling molecules continually emerge [33-35] and may ultimately clarify the role of PMCA in regulating signalling events in platelets.

Altered expression of PMCA4b has been reported in patients with diabetes [36], hypertension [37] and type 2B von Willebrand disease [38]. All of these conditions are associated with platelet dysfunction through mechanisms that are incompletely understood. In defining the
role of PMCA in regulating platelet function, we provide potential mechanisms by which
PMCA may modulate platelet function and hence the development and progression of these
diseases.

In conclusion, PMCA is involved in the regulation of \([\text{Ca}^{2+}]_{i}\), in both resting and activated
platelets. PMCA differentially regulates the various stages of platelet activation with both
stimulatory and inhibitory roles. The effects on platelet function correspond with changes in
both \([\text{Ca}^{2+}]_{i}\) and inhibitory signalling pathways. PMCA is clearly a critical regulator of
platelet Ca\(^{2+}\) homeostasis, signalling and function and is therefore a key regulator of
haemostasis and thrombosis.
Acknowledgements

This work was funded by a Research Grant from the Wellcome Trust (085132/Z/08/Z) to ME. LN is supported by an MRC Programme Grant and by NIHR (Manchester Biomedical Research Centre). AS is supported by a National Heart and Lung Institute Foundation Studentship.

Disclosure of Conflict of Interests

The authors declare that they have no conflict of interest.
References


**Figure Legends**

**Fig. 1.** Carboxyeosin inhibits Ca^{2+} extrusion through PMCA in platelets and modifies Ca^{2+} homeostasis. Washed platelets loaded with Fura2 were incubated for 30 min with the PMCA inhibitor carboxyeosin (CE; 10-40 µM) or DMSO (0.4%, Control) and [Ca^{2+}], measured as 340/380 emission ratio (F 340/380). (A) Platelets were stimulated with thapsigargin (Tg, 1 µM) supplemented with ionomycin (Iono, 50 nM) to induce Ca^{2+} release followed by extrusion through PMCA. CE pre-treatment impaired Ca^{2+} extrusion shown as an impaired return to baseline. Ca^{2+} release from stores is indicated by arrows for control and 40 µM CE treatments and show that Ca^{2+} release is not affected by CE. Typical smoothed traces of n = 5 are shown. (B) Mean [Ca^{2+}]i in resting platelets. (C) Mean maximal [Ca^{2+}]i following stimulation with collagen (5 µg/ml) for 180s. (D) Time course of 340/380 ratio (F 340/380) in platelets stimulated with collagen for 30 minutes, showing a typical trace of n = 5. Data represent mean ± s.e.m. (n = 4), *p<0.05, **p<0.01.

**Fig. 2.** Inhibition of PMCA inhibits platelet aggregation and secretion. Washed human platelets were incubated with the PMCA inhibitor carboxyeosin (CE) (10-40 µM) or DMSO (0.4%) for 30 min prior to stimulation with collagen (5 µg/ml) and (A) aggregation measured using optical aggregometry (n = 4) or (B) [\textsuperscript{3}H]5-HT release measured from [\textsuperscript{3}H]5-HT pre-loaded platelets. (C) Representative aggregometry traces and (D) mean maximal aggregation responses of platelets from wild-type (WT) and PMCA4\textsuperscript{−/−} mice following stimulation with collagen (0.5 µg/ml). (E-F) Representative aggregation traces following collagen (E) or thrombin (F) in the presence of CE (40 µM) in platelets from WT and PMCA4\textsuperscript{−/−} mice. Data represent mean ± s.e.m. (n = 4), *p<0.05.
Fig. 3. PMCA inhibition reduces platelet responses in vivo. Washed platelets labelled with $^{111}$Indium oxine and incubated with the PMCA inhibitor carboxyeosin (CE, 20 or 40 µM) or DMSO control (0.4%) were infused into anaesthetized recipient mice, which were subsequently injected with collagen (25, 50 and 75 µg/kg i.v.). Radiolabelled platelet aggregation in the pulmonary vasculature was measured via external detection probes. Data are shown as (A) changes in counts over time or (B) mean ± s.e.m of the maximal % increase in $^{111}$Indium counts compared to basal levels, n = 5, *p<0.05, **p<0.01.

Fig. 4. PMCA inhibition enhances platelet adhesion and clot retraction. Washed platelet adhesion to fibrinogen (100 µg/ml) was measured under static conditions in platelets pre-incubated for 30 min with the PMCA inhibitor carboxyeosin (CE, 10-40 µM) (n = 3). (A) Clot retraction induced by thrombin (2.5 U/ml) was measured in PRP pre-incubated for 30 min with CE (20 or 40 µM), (B) images of the clots were taken after 2 hours and (C) mean clot weights expressed relative to controls. Data are representative of mean ± s.e.m., n = 3, *p<0.05, **p<0.01.

Fig. 5. PMCA enhances VASP phosphorylation independently of cGMP. Platelet lysates from collagen (5 µg/ml) stimulated platelets pre-incubated with CE (10-40 µM) were (A) immunoblotted for phospho-VASP (Ser239) or total VASP and (B) phospho-VASP levels normalised. (C) platelet aggregation was measured in collagen (5 µg/ml) stimulated platelets pre-incubated with carboxyeosin (CE, 20 µM) or sodium nitroprusside (SNP) (1 µM) in the
presence and absence of ODQ (10 µM). Data represent mean ± s.e.m, n = 3, *p<0.05, **p<0.01, ***p<0.001.
Fig. 1. Carboxyeosin inhibits Ca2+ extrusion through PMCA in platelets and modifies Ca2+ homeostasis. Washed platelets loaded with Fura2 were incubated for 30 min with the PMCA inhibitor carboxyeosin (CE; 10-40 □M) or DMSO (0.4%, Control) and [Ca2+]i measured as 340/380 emission ratio (F 340/380). (A) Platelets were stimulated with thapsigargin (Tg, 1 □M) supplemented with ionomycin (Iono, 50 nM) to induce Ca2+ release followed by extrusion through PMCA. CE pre-treatment impaired Ca2+ extrusion shown as an impaired return to baseline. Ca2+ release from stores is indicated by arrows for control and 40 □M CE treatments and show that Ca2+ release is not affected by CE. Typical smoothed traces of n = 5 are shown. (B) Mean [Ca2+]i in resting platelets. (C) Mean maximal [Ca2+]i following stimulation with collagen (5 □g/ml) for 180s. (D) Time course of 340/380 ratio (F 340/380) in platelets stimulated with collagen for 30 minutes, showing a typical trace of n = 5. Data represent mean ± s.e.m. (n = 4), *p<0.05, **p<0.01.
Fig. 2. Inhibition of PMCA inhibits platelet aggregation and secretion. Washed human platelets were incubated with the PMCA inhibitor carboxyeosin (CE) (10-40 µM) or DMSO (0.4%) for 30 min prior to stimulation with collagen (5 µg/ml) and (A) aggregation measured using optical aggregometry (n = 4) or (B) [3H]5-HT release measured from [3H]5-HT pre-loaded platelets. (C) Representative aggregometry traces and (D) mean maximal aggregation responses of platelets from wild-type (WT) and PMCA4-/- mice following stimulation with collagen (0.5 µg/ml). (E-F) Representative aggregation traces following collagen (E) or thrombin (F) in the presence of CE (40 µM) in platelets from WT and PMCA4-/- mice. Data represent mean ± s.e.m. (n = 4), *p<0.05.
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Fig. 3. PMCA inhibition reduces platelet responses in vivo. Washed platelets labelled with [111]Indium oxine and incubated with the PMCA inhibitor carboxyeosin (CE, 20 or 40 µM) or DMSO control (0.4%) were infused into anaesthetized recipient mice, which were subsequently injected with collagen (25, 50 and 75 µg/kg i.v.). Radiolabelled platelet aggregation in the pulmonary vasculature was measured via external detection probes. Data are shown as (A) changes in counts over time or (B) mean ± s.e.m of the maximal % increase in [111]Indium counts compared to basal levels, n = 5, *p<0.05, **p<0.01.

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Fig. 4. PMCA inhibition enhances platelet adhesion and clot retraction. Washed platelet adhesion to fibrinogen (100 µg/ml) was measured under static conditions in platelets pre-incubated for 30 min with the PMCA inhibitor carboxyeosin (CE, 10-40 µM) (n = 3). (A) Clot retraction induced by thrombin (2.5 U/ml) was measured in PRP pre-incubated for 30 min with CE (20 or 40 µM), (B) images of the clots were taken after 2 hours and (C) mean clot weights expressed relative to controls. Data are representative of mean ± s.e.m., n = 3, *p<0.05, **p<0.01.
Figure 5. PMCA enhances VASP phosphorylation independently of cGMP. Platelet lysates from collagen (5 µg/ml) stimulated platelets pre-incubated with CE (10−40 µM) were (A) immunoblotted for phospho-VASP (Ser239) or total VASP and (B) phospho-VASP levels normalised. (C) platelet aggregation was measured in collagen (5 µg/ml) stimulated platelets pre-incubated with carboxyeosin (CE, 20 µM) or sodium nitroprusside (SNP) (1 µM) in the presence and absence of ODQ (10 µM). Data represent mean ± s.e.m, n = 3, *p<0.05, **P<0.01, ***P<0.001.
Supplementary Figure Legends

Supplementary Fig. 1. Carboxyeosin does not affect stored Ca^{2+} release. Stored Ca^{2+} release was measured as peak response following thapsigargin plus ionomycin minus stable baseline values in Fura2 loaded platelets pretreated with carboxyeosin (10-40 µM) or DMSO control. Data represent mean ± s.e.m. (n = 5). No significant changes were observed.

Supplementary Fig. 2. Effect of eosin and carboxyeosin on Fura2 emission spectra. Mean stable 340/380 ratio was measured over a 2 min period. All experiments were conducted in dH_{2}O containing 100 nM CaCl_{2}. Blank readings were taken in dH_{2}O (dH_{2}O(c)) and with DMSO (0.1 %) (DMSO(c)) and then basal emission measured in the presence of 0.5 µM Fura2 with (A) eosin (Eo, 0.1 – 50 µM) or (B) carboxyeosin (CE, 0.1 – 50 µM) or vehicle control (Ca(c)) in the presence of Ca^{2+}. Data are expressed as mean ± S.E.M. (n = 5), no significant changes were observed.

Supplementary Fig. 3. Effect of blockade of Ca^{2+} extrusion on platelet Ca^{2+} homeostasis. Fura2 loaded platelets were pre-treated with lanthanum (LaCl_{3}, 1 mM), carboxyeosin (CE, 40 µM) or DMSO (CONTROL) prior to stimulation with thapsigargin plus ionomycin (Tg + Iono, 1 µM + 50 nM). Responses were compared with platelets that were treated with carboxyeosin following lanthanum (CE + LaCl_{3}).

Supplementary Fig. 4. Na^+/K^+-ATPase does not modulate platelet aggregation. Isolated human platelets were pre-incubated with the Na^+/K^+-ATPase inhibitor ouabain (0.01 – 200 µM) or vehicle (dH_{2}O) control for 5 min prior to stimulation with
(A) collagen (5 µg/ml) or (B) thrombin (0.1 U/ml). Platelet aggregation was expressed relative to control responses. Data are expressed as mean ± S.E.M. (n = 4), no significant changes were observed.

Supplementary Fig. 5. Carboxyeosin inhibits aggregation of human and mouse platelets. (A) Washed human platelets were pretreated with carboxyeosin (CE, 10-40 µM) or DMSO and aggregation assessed in response to thrombin (0.1 U/ml); (B) effect of carboxyeosin on collagen (5 µg/ml) induced aggregation of washed mouse platelets.

Supplementary Fig. 6. Increased [Ca^{2+}] after PMCA inhibition does not desensitize platelets. Platelets were incubated with (A-B) thapsigargin (0.25-5 µM) or (C-D) carboxyeosin (CE, 10-40 µM) for 30 min and [3H]5-HT release measured from pre-loaded unstimulated platelets (A,C) or light transmission through unstimulated platelet suspensions measured using optical aggregometry (B,D). Data represent mean ± s.e.m. (n = 3), *p<0.05, **p<0.01.
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