**Calcium sequestration by human platelet acidic organelles is regulated by the actin cytoskeleton and autocrine 5-hydroxytryptamine**

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Abstract

Human platelets regulate agonist-evoked Ca2+ signalling through Ca2+ release from and sequestration into acidic organelles. Previous studies have pharmacologically characterised the presence of a Ca2+-H+ exchanger in these organelles. This exchanger appears to regulate a secondary plateau phase in agonist-evoked cytosolic Ca2+ signals in fura-2-loaded human platelets. Here we demonstrate that cytochalasin D treatment removes the secondary plateau in ADP-evoked Ca2+ signals elicited in the absence of external Ca2+. This effect was reversed by pretreatment with nigericin, a K+/H+ exchanger that short-circuits the Ca2+-H+ exchanger. Using Fluo-5N- or Lysosensor Green-loaded cells, cytochalasin D was found to enhance Ca2+ sequestration into acidic organelles by preventing their alkalinisation. Additional experiments demonstrated that ADP-evoked alkalinisation of acidic organelles and subsequent slowing of acidic organellar Ca2+ sequestration was mediated by autocrine 5-HT signalling. Enhancing this 5-HT signalling using fluoxetine overcame the inhibitory effect of cytochalasin D on ADP-evoked Ca2+ signals, indicating that cytochalasin D interferes with 5-HT autocrine signalling. The ability of Cytochalasin D to interfere with autocrine 5-HT signalling was downstream of the 5-HT2A receptor as secretion of [3H]-5-HT from ADP-stimulated human platelets was not reduced. These data provide the first evidence that the pH gradient across acidic organelles is dynamically regulated upon human platelet activation, and that this can play a significant role in controlling human platelet function by modulating Ca2+-H+ exchange and so [Ca2+]i.

**Keywords: Human platelet, acidic organelle, calcium sequestration, pH, ADP, 5-hydroxytrptamine**

**Introduction**

A rise in platelet cytosolic Ca2+ concentration ([Ca2+]i) is essential for human platelet activation and so involvement in thrombus formation [1]. This Ca2+ signal is generated by both Ca2+ entry across the plasma membrane and Ca2+ release from intracellular stores [1].

Platelets possess at least two agonist-releasable Ca2+ stores [2] that are differentially regulated. The larger store is located in the dense tubular system (DTS), is filled by the thapsigargin-sensitive sarco/endoplasmic reticulum Ca2+-ATPase 2b (SERCA2b) [3] and is released by agonists via the formation of inositol 1,4,5-trisphosphate (IP3) [4]. Another, smaller Ca2+ store is located in platelet acidic organelles [5], which include lysosomes [6], alpha granules [7, 8] and dense granules [9]. Ca2+ release from acidic organelles occurs via activation of nicotinic acid-adenine dinucleotide phosphate (NAADP) receptors in human platelets [5, 6, 10] and other cells [11]. However, in platelets this release is differentially controlled by different agonists: thrombin and collagen, but not ADP or arginine vasopressin, are able to release Ca2+ from the acidic organelles via NAADP formation [5, 6, 10].

Although not all platelet agonists release Ca2+ from acidic organelles, Ca2+ sequestration into acidic organelles has been shown to modulate Ca2+ signals elicited by all platelet agonists studied [6, 12]. This Ca2+ sequestration appears to have no impact on the initial Ca2+ spike generated by agonist-evoked Ca2+ release, but instead modulates a later secondary plateau phase of the cytosolic Ca2+ signal [6, 12]. However, the mechanisms underlying Ca2+ sequestration into platelet acidic Ca2+ stores are currently not fully delineated. SERCA3 plays a role in refilling the acidic Ca2+ stores and modulating agonist-evoked cytosolic Ca2+ signals in platelets [6, 13]. However, it has also been demonstrated that sequestration of Ca2+ into platelet acidic stores is dependent upon the activity of a vacuolar-type H+ ATPase (v- H+-ATPase) coupled to a putative Ca2+- H+ exchanger [5, 12]. The activity of the Ca2+-H+ exchanger can be prevented by treatment of platelets with the K+/H+ exchanger, nigericin. Nigericin acts to prevent Ca2+-H+ exchange by collapsing the pH gradient between the interior of the acidic organelle and the cytosol. Despite this pharmacological characterisation, there is currently no evidence for the molecular identity of the transport protein(s) that mediate Ca2+-H+ exchange and it is not known whether this sequestration mechanism is modulated upon agonist stimulation of human platelets.

As ADP does not trigger NAADP-evoked Ca2+ release from the intracellular stores in platelets, but the Ca2+ signal is modulated by Ca2+ sequestration into the acidic organelles, examination of Ca2+ signals in response to this agonist provides a model system in which to explore the regulation of platelet acidic organelle Ca2+ sequestration. Any experimental manipulation that specifically modulates the magnitude or duration of the secondary plateau phase of the cytosolic Ca2+ signal, without affecting the magnitude of the initial IP3-triggered Ca2+ spike, could indicate a cellular mechanism that regulates Ca2+-H+ exchange across the acidic organelle membrane. Previously we have demonstrated that the secondary plateau phase of ADP-evoked Ca2+ signals evoked in the absence of extracellular Ca2+ is significantly reduced when platelets are pre-treated with inhibitors of actin polymerisation [14] or autocrine 5-HT signalling [15, 16]. These data suggest the possibility that both reorganisation of the actin cytoskeleton and autocrine 5-HT signalling play a key role in regulating Ca2+ uptake into acidic organelles. Here we confirm that the actin cytoskeleton and autocrine 5-HT are important in regulating acidic store Ca2+ sequestration in human platelets by a mechanism involving alteration of the pH gradient between the acidic store lumen and the cytosol, thus demonstrating for the first time that Ca2+-H+ exchange into the acidic organelles is actively modulated by intracellular signalling pathways.

**Materials and Methods**

**Materials**

Fluoxetine was from Axxora (Nottingham, UK). Apyrase, adenosine diphosphate (ADP), bovine serum albumin (BSA), nigericin, 2,5-di(tert-butyl) l,4-benzohydroquinone (TBHQ) and thapsigargin were from Sigma Aldrich (Gillingham, UK). Fura-2/AM and BCECF/AM were from TEFlabs (Austin, TX, USA). Fluo-5N/AM and Lysosensor Green DND-189 were from Invitrogen (Paisley,UK). Cytochalasin D, ketanserin and RGDS peptide were from Tocris Cookson (Bristol, UK). All other reagents were of analytical grade.

**Platelet Preparation**

Platelets were prepared as described previously [17]. Briefly, blood was collected by venepuncture from healthy drug-free volunteers under informed consent with local ethical committee approval, and then mixed with one-sixth volume acid-citrate dextrose (ACD) anticoagulant (85 mM sodium citrate, 78 mM citric acid, and 111 mM D-glucose). Platelet-rich plasma (PRP) was prepared by centrifugation for 5 min at 700 *g*, and aspirin (100 μM) and apyrase (0.1 U mL-1) added. Washed platelets were prepared by centrifugation of PRP at 350 *g* for 20 min and resuspended in HEPES-buffered saline (HBS; 145 mM NaCl, 10 mM HEPES, 10 mM D-glucose, 5 mM KCl, 1 mM MgSO4, pH 7.45) supplemented with 0.1% (w/v) BSA, 200 μM CaCl2 and 0.1 U mL-1 apyrase (supplemented HBS).

#### Indicator loading

#### For measurement of cytosolic Ca2+ concentration ([Ca2+]i), intracellular store Ca2+ concentration ([Ca2+]st), cytosolic pH (pHi) or acidic organelle pH (pHao,), platelets were loaded with Fura-2, Fluo-5N, BCECF or Lysosensor Green DND-189, respectively. Fura-2, Fluo-5N or Lysosensor Green DND-189 were loaded by incubating platelet-rich plasma with either 2.5 μM Fura-2/AM for 45 min, 250 nM Fluo-5N/AM for 2 hours or 200 nM Lysosensor Green DND-189 for 1 hour at 37 °C. Cells were then collected by centrifugation at 350 *g* for 20 min and resuspended in supplemented HBS. RGDS peptide (100 µM) was included in all experiments with Fluo-5N- or Lysosensor Green DND-loaded cells to prevent aggregation and therefore artefactual decreases in fluorescence. RGDS at this concentration has previously been shown not to affect [Ca2+]i signals in human platelets [18].

#### BCECF was loaded by incubating washed platelet suspension with 1 µM BCECF/AM for 30 min at room temperature. 10% v/v ACD was then added before the cells were recollected by centrifugation in a minifuge at 8000 *g* for 30 s. The platelets were then resuspended in supplemented HBS.

#### Measurement of [Ca2+]i, [Ca2+]st, pHi and pHao

Fluorescence was recorded from 1.5 ml aliquots of stirred platelet suspension (approximately 3 x 108 cells/ml) at 37 °C using a Cairn Research Spectrophotometer (Cairn Research, Faversham, UK) with excitation wavelengths of 340 and 380 nm (Fura-2) or 485 nm (Fluo-5N) and emission at 515 nm. Changes in [Ca2+]i were monitored using the Fura-2 340/380 fluorescence ratio and calibrated according to Grynkiewicz et al. [19]. [Ca2+]i was quantified by integration of the agonist-evoked change in [Ca2+]i  from basal levels with respect to time for 60 s after the addition of ADP. Fluo-5N fluorescence was similarly quantified. Basal levels were taken as the mean of the [Ca2+]i or [Ca2+]st (uncalibrated Fluo-5N fluorescence) for 10 s prior to the addition of agonists. In the experiments using nigericin, Fluo-5N fluorescence was corrected for the small autofluorescence of the compound by subtracting the increase in fluorescence observed when adding nigericin to a sample of supplemented HBS alone.

BCECF fluorescence was recorded and quantified as for Fura-2 above but with excitation wavelengths of 440 and 480 nm and emission at 515 nm. pHi was monitored as the 480/440 nm fluorescence ratio.

Lysosensor Green DND-189 fluorescence was recorded and quantified as for Fluo-5N above but with excitation at 445 nm and emission at 515 nm.

**5-HT secretion**

Platelets were loaded with [3H]-5-HT by adding 16.5 µCi of [3H]-5-HT to 25 ml of PRP prepared as above. After incubation for 1 hour at 37 °C, the cells were collected by centrifugation and resuspended in 45 ml of HBS. 4.5 ml aliquots of this suspension were then incubated with 10 µM Cyt D or its vehicle, DMSO, for 40 min at 37ºC before the addition of fluoxetine (20 µM) or its vehicle, DMSO, immediately before the cells were stimulated with ADP (50 µM), or its vehicle, HBS, was added. The suspension was maintained at 37 °C and 0.4 ml aliquots were withdrawn in duplicate immediately before stimulation and at 15, 30, 60 and 180 s after stimulation. The cells were then fixed by the addition of 0.4 ml of 3% [w/v] paraformaldehyde. The platelets were then removed by centrifugation in a minifuge at 8000 *g* for 30 s and 0.4 ml aliquots of the supernatant taken for counting after addition of scintillant (Bioscint, National Diagnostics). Counting was conducted using a LKB Wallac 1216 Liquid Scintillation Counter.

**Results**

**Disruption of the actin cytoskeleton using cytochalasin D abolishes a secondary plateau phase in the ADP-evoked rise in [Ca2+]i mediated by 5-HT autocrine signalling via dense granule secretion**

Pre-treatment of platelets with cytochalasin D (Cyt D; 10 µM) for 40 min at 37ºC has previously been shown to completely inhibit agonist-evoked actin polymerisation [20]. We have also reported that pre-treating human platelets in this manner inhibited a secondary plateau phase but not the initial spike in [Ca2+]i evoked by ADP (50 µM) in the absence of extracellular Ca2+ (1 mM EGTA added) [14]. Previously we have demonstrated that the inhibitor of Na+/Ca2+ exchange, KB-R7943, inhibited a secondary component of thrombin-evoked rises in [Ca2+]i elicited in the absence of external Ca2+ [21]. This was found to be mediated in part by autocrine signalling. To investigate if the delayed phase of the ADP-evoked Ca2+ signal is dependent on NCX activity and dense granule secretion, we examined the effect of KB-R7943, an inhibitor of Na+/Ca2+ exchange that inhibits platelet dense granule secretion and so 5-HT release [16, 21]. As shown in Figure 1A, Cyt D inhibited the secondary plateau phase of the ADP-evoked rise in [Ca2+]i and reduced the ADP-evoked Ca2+ signal to 70.9 ± 7.7% of vehicle (DMSO)-treated control (Fig. 1A; n = 5, P < 0.05) and pre-treatment of cells with KB-R7943 (50 µM) for 2 min reduced the ADP-evoked response to 43.6 ± 2.2% of control (n = 5, P < 0.05).

Previous studies have also indicated that the secondary plateau phase of the ADP-evoked rise in [Ca2+]i was mediated by autocrine 5-HT signalling, since it was eliminated in the presence of the 5-HT2A receptor antagonist, ketanserin [15]. We therefore investigated whether alterations in autocrine 5-HT signalling could be responsible for altering the Ca2+ release observed in Cyt D-treated cells stimulated with ADP. To do this we examined whether pretreatment of platelets with fluoxetine, an inhibitor of the 5-HT transporter, SERT [22], could reverse the effect of Cyt D and KB-R7943 on ADP-evoked changes in [Ca2+]i. It has been previously demonstrated by our group and others that fluoxetine-treatment significantly potentiates ADP-evoked Ca2+ signals in human platelets through preventing 5-HT reuptake and thus enhancing autocrine 5-HT signalling [15, 23]. Addition of fluoxetine immediately before ADP stimulation abolished the effect of Cyt D on the ADP-evoked Ca2+ signal, which was 107.5 ± 7.0% of control (Fig. 1B; P > 0.05; n = 7). In contrast, fluoxetine did not reverse the inhibitory effect of KB-R7943 (Fig. 1B; 56.3 ± 4.4% of control; P < 0.05, n = 7), which inhibits 5-HT release by preventing dense granule secretion [16]. The ability of fluoxetine to reverse the action of Cyt D on the ADP-evoked cytosolic Ca2+ signal confirms that the actin polymerisation inhibitor interferes with the autocrine 5-HT signalling that underlies the secondary plateau phase of the cytosolic Ca2+ signal. The effect of Cyt D pre-treatment on the ADP-evoked Ca2+ signal might be explained by reduced 5-HT secretion, increased 5-HT reuptake, altered signalling downstream of the 5-HT2A receptor, or a combination of these.

As KB-R7943 has been previously shown to inhibit dense granule secretion from human platelets, we examined whether the effects of Cyt-D were additive with the effects of KB-R7943. Consistent with these previous findings, positive control experiments demonstrated that pretreatment with Cyt D reduced the ADP-evoked Ca2+ signal to 84.8 ± 0.3% of vehicle (DMSO)-treated control (Fig. 2A). In the presence of KB-R7943, Cyt D pre-treatment was without additional effect (Fig. 2B); the ADP-evoked Ca2+ signal was 108.1 ± 20.6% of that observed in cells treated with KB-R7943 alone (Fig. 2B, P > 0.05, n = 6). Taken together the above results indicate that Cyt D inhibition of autocrine 5-HT signalling is not due to inhibition of dense granule secretion. This is consistent with previous findings that demonstrated that pre-treatment with actin polymerisation inhibitors enhance agonist-evoked dense granule secretion in human platelets [24].

To examine this further we used a [3H]-5-HT secretion assay to investigate the effect of Cyt D and fluoxetine alone and in combination on 5-HT release into the extracellular fluid. As shown in Fig. 3, stimulation with ADP increased extracellular 5-HT compared with unstimulated control cells. Pre-treatment of the cells with Cyt D enhanced this extracellular 5-HT accumulation, consistent with the results of previous studies demonstrating enhanced dense granule secretion after treatment with inhibitors of actin polymerisation [24, 25]. Furthermore, inhibiting SERT with fluoxetine enhanced the ADP-evoked increase in extracellular 5-HT as expected in control cells, and this increase was further enhanced when fluoxetine-treated cells were also pre-treated with Cyt D (Fig. 3). Hence reduced secretion or enhanced reuptake of 5-HT across the platelet plasma membrane cannot account for the reduction in autocrine 5-HT signalling following Cyt D pre-treatment. These results therefore indicated that Cyt D treatment affects signalling downstream of the plasma membrane 5-HT2A receptor.

**Cytochalasin D inhibits the secondary plateau phase of the ADP-evoked rise in [Ca2+]i by enhancing Ca2+ sequestration into acidic organelles**

The initial ADP-evoked spike in [Ca2+]i evoked in the absence of extracellular Ca2+ principally results from IP3-mediated release of Ca2+ from the DTS [4, 26], whilst the secondary plateau phase is regulated by Ca2+ sequestration into acidic organelles [12]. To investigate if Cyt D reduces the secondary phase of ADP-evoked Ca2+ release by enhancing Ca2+-H+ exchange across acidic organelle membranes we examined whether nigericin could reverse the inhibitory effect of the actin polymerisation inhibitor. Nigericin collapses H+ gradients throughout platelets by acting as a K+/H+ antiporter, preventing Ca2+ reuptake into acidic organelles. As described above, in the absence of nigericin, cytochalasin D significantly reduced the ADP-evoked Ca2+ signal (Fig. 4A; 85.5 ± 2.8% of control; P < 0.05, n = 8). Simultaneous addition of nigericin (10 µM) with ADP (50µM) potentiated the ADP-evoked Ca2+ signal to 179.5 ± 12.6% of vehicle (ethanol)-treated control (Fig. 4A; n = 8, P < 0.05), presumably due to inhibition of Ca2+-H+ exchange across the acidic organelles. However, when platelets were stimulated concomitantly with both ADP and nigericin, Cyt D pre-treatment had no effect on this response (Fig. 4A; 95.5 ± 3.7% of DMSO-treated control; P > 0.05, n = 8). This result suggests that the actin cytoskeleton plays a role in patterning the ADP-evoked Ca2+ signal by regulating Ca2+ sequestration into acidic organelles. Ca2+ sequestration by Ca2+-H+ exchange would be eliminated when the H+ gradient across acidic organelle membranes is collapsed by nigericin.

Experiments were performed in Fluo-5N-loaded human platelets to assess if nigericin was able to reverse the effect of Cyt D on the net Ca2+ fluxes from intracellular stores. Fluo-5N is a low-affinity fluorescent Ca2+ indicator that has previously been shown to selectively monitor changes in the Ca2+ concentration of platelet intracellular stores ([Ca2+]st) [12]. As previously observed, stimulating Fluo-5N-loaded platelets with 50 µM ADP in the absence of extracellular Ca2+ evoked a decrease in [Ca2+]st that was followed by partial refilling over about 30 s (Fig. 4B). After pre-treatment with Cyt D, the initial Ca2+ release was similar to that seen in control (DMSO-treated) cells, but refilling occurred more rapidly and to a greater extent (corresponding to the reduction in [Ca2+]i; Fig. 4A). Cyt D reduced the integral of the ADP-evoked decrease in [Ca2+]st to 39.3 ± 11.6% of control (P < 0.05, n = 6) over the 60 s following ADP addition. However, in the presence of nigericin, Cyt D pre-treatment was without effect on the ADP-evoked decrease in [Ca2+]st (Fig. 4C and Figure S1 (supplementary materials); 87.4 ± 15.8% of control; P > 0.05, n = 6). These data support the hypothesis that Cyt D inhibits the secondary phase of the ADP-evoked rise in [Ca2+]i observed in the absence of extracellular Ca2+ by enhancing the rate of Ca2+ sequestration into acidic organelles via Ca2+-H+ exchange. When this exchange mechanism is inhibited by concomitant treatment with nigericin, Cyt D has no significant effect on the ADP-evoked increase in [Ca2+]i or decrease in [Ca2+]st.

**Cyt D enhances Ca2+ sequestration by inhibiting the ADP-evoked alkalinisation of acidic organelles.**

To investigate whether Cyt D altered Ca2+-H+ exchange by modulating the pH gradient between the lumen of acidic organelles and the cytosol, we monitored changes in acidic organelle pH (pHao) using the indicator Lysosensor Green DND-189. This indicator

loads into acidic compartments and emits decreasing fluorescence with a rise in pH [5]. ADP evoked a sustained decrease in Lysosensor Green DND-189 fluorescence indicating a rise in pHao (Fig. 5A). After treatment with Cyt D, ADP initially evoked a rise in pHao at a similar rate to that observed in controls, but after about 15s pHao started to fall again (Fig. 5A), at a time corresponding to the secondary phase of the ADP-evoked rise in [Ca2+]i (Fig. 1A). Over the 60 s from stimulation Cyt D pre-treatment reduced the ADP evoked rise in pHao to 55.4 ± 6.2% of control (Fig. 5A; n = 6, P < 0.05). The sustained acidic organelle alkalinisation evoked by ADP under control conditions would reduce Ca2+ sequestration by Ca2+-H+ exchange, so producing a plateau in the rise in [Ca2+]i, itself caused by Ca2+ release from the DTS. The early reacidification after ADP stimulation observed in Cyt D pre-treated cells could result in increased Ca2+ sequestration by Ca2+/H+ exchange into the acidic organelles, so eliminating the plateau in [Ca2+]i. Given the time delay after agonist-evoked stimulation, this would appear to be an event regulated by cellular signalling. The data in Figure 5A also demonstrate that alkalinisation of the acidic organelles is the cause of the reduced cytosolic Ca2+ signal, and not a consequence of it. If the effect of Cyt D was to directly enhance Ca2+/H+ exchange into the acidic organelles, then this would be expected to potentiate, rather than inhibit, the alkalinisation of the acidic organelles, thus demonstrating that Cyt D has an indirect, pH-dependent effect on Ca2+ sequestration into the acidic organelles.

Simultaneous addition of nigericin with ADP potentiated the ADP-evoked rise in pHao (Fig. 5B; 278.3 ± 46.7% of control, n = 6, P < 0.05). The sustained rise in pHao would be expected to reduce acidic organelle Ca2+ sequestration by Ca2+-H+ exchange, which could in turn be responsible for the augmented initial spike and plateau in [Ca2+]i in the presence of nigericin (Fig. 4A). In the presence of nigericin, Cyt D pre-treatment slowed and reduced the extent of the ADP-evoked rise in pHao (Fig. 5B; 73.0 ± 5.5% of nigericin-treated control; n = 6, P < 0.05), although this alkalinisation was maintained and there was no reacidification as seen in the absence of nigericin (Fig. 5A). In the presence of high concentrations of the K+/H+ exchanger nigericin, the acidic organelle H+ permeability would be significantly enhanced and would largely negate any difference in acidic organelle H+ permeability between control- and Cyt D pre-treated platelets. Therefore, it is unlikely that Cyt D inhibition of agonist-evoked changes in the H+ permeability of acidic organelles underlie this effect. This suggests that the fall in pHao after the initial ADP-evoked rise observed in Cyt D treated cells is most likely due to enhanced H+ reuptake rather than decreased H+ release.

To confirm that the alkalinisation of the acidic organelles was due to H+ flux between the lumen of the organelles and the cytosol, we monitored cytosolic pH (pHi) in BCECF-loaded platelets [27]. BCECF fluorescence decreases with a fall in pHi, which is predicted to occur when protons are released from acidic organelles during organelle alkalinisation. As shown in Fig. 5C, ADP evoked a decrease in pHi as previously reported [27]. This ADP-evoked fall in pHi was reduced in platelets pre-treated with Cyt D (Fig. 5C, 35.6 ± 10.8% of control; n = 6, p < 0.05), consistent with the observed reduction in the ADP-evoked rise in pHao. Nigericin accelerated and increased the extent of the ADP-evoked decrease in pHi, which then rapidly recovered towards basal values (Fig. 5D, 232.2 ± 56.4% of control; n = 6, P < 0.05). Although Cyt D pre-treatment was without significant effect on the integral of the change in BCECF fluorescence over the 60 s from ADP stimulation in the presence of nigericin (Fig. 5D, 94.9 ± 4.4% of nigericin-treated control; n = 6, P > 0.05), the rise in pHi occurred more rapidly,indicating a faster clearance of H+ from the cytosol after Cyt D pre-treatment resulting in a reduced mean integral with less variance in Cyt D-treated cells exposed to nigericin, compared to cells treated with both DMSO and nigericin (Supplementary Materials, Figure S2). As this mirrors the enhanced acidification of the acidic organelles, these data support the hypothesis that disrupting the actin cytoskeleton using Cyt D enhances H+ uptake into acidic organelles. These data also demonstrate that the alkalinisation observed was not due to secretion of the luminal contents of the acidic secretory granules into the extracellular space, as in this scenario, changes in the cytosolic pH would not mirror the changes observed in the acidic organelles.

**5-HT signalling is required for ADP-evoked alkalinsation of the acidic organelles**

As we have shown above that Cyt D impacts on ADP-evoked alkalinisation of the acidic organelles, we considered whether autocrine 5-HT signalling might be responsible for the acidic organelle alkalinisation that slows Ca2+ sequestration to produce the secondary plateau phase in the [Ca2+]i signal. If Cyt D reduces the efficiency of this autocrine 5-HT signalling pathway, increased external 5-HT signalling elicited by fluoxetine pre-treatment may be able to reverse the effect of Cyt D. Therefore we investigated the effects of modulators of 5-HT signalling on ADP-evoked changes in [Ca2+]st, pHao and pHi to assess whether this signalling pathway is responsible for the ADP-evoked pH changes in the acidic organelle, and the subsequent reuptake of Ca2+ into these acidic Ca2+ stores. Addition of fluoxetine (20 µM) immediately before the start of experiments enhanced the ADP-evoked changes in [Ca2+]st, pHao and pHi to 166.1 ± 23.8% of control (Fig. 6A; P < 0.05, n = 5), 186.7 ± 20.1% of control (Fig. 6B; P < 0.05, n = 7) or 186.1 ± 30.7% of control (Fig. 6C; P < 0.05, n = 6), respectively. These results indicate that the enhanced autocrine 5-HT signalling elicited by fluoxetine is responsible for enhancing the alkalinisation of the acidic organelle, caused by reducing H+ reuptake from the cytosol. This in turn reduces the reuptake of Ca2+ into the acidic organelles by reducing Ca2+-H+ exchange.

To assess if reducing autocrine 5-HT signalling has the converse effect, we assessed whether addition of the 5HT2A receptor antagonist ketanserin could block the ADP-evoked alkalinisation of the acid stores and enhance Ca2+ reuptake. Addition of ketanserin (50 µM) 2 min before the start of experiments reduced ADP-evoked changes in [Ca2+]st, pHao and pHi to 31.1 ± 6.2% of control (Fig. 6D; P < 0.05, n = 5), 56.9 ± 14.3% of control (Fig. 6E; P < 0.05, n = 7) or 59.4 ± 7.3% of control (Fig. 6F; P< 0.05, n = 6), respectively. These results are consistent with the previously reported abolition of the secondary phase of the ADP-evoked rise in [Ca2+]i in the presence of ketanserin [15]. However, in this study we demonstrate for the first time this is due to a pH dependent effect on Ca2+ sequestration into the acidic organelles.

**Discussion**

In this study we demonstrate for the first time that Ca2+ sequestration into platelet acidic organelles is actively regulated by autocrine 5-HT signalling in a manner that is dependent upon the integrity of the actin cytoskeleton. The selective impact of Cyt D pre-treatment on the secondary plateau phase of ADP-evoked rises in [Ca2+]i in the absence of extracellular Ca2+ suggests that this plateau is mediated by regulation of Ca2+ sequestration into the acidic organelles. As ADP is without effect on the initial spike in the Ca2+ signal this effect is not due to an altered IP3 formation, activation of the IP3 receptor or activation of SERCA2b. As the effect of Cyt D on ADP-evoked increases in [Ca2+]i and decreases in [Ca2+]st can be reversed by treatment with nigericin, the plateau in [Ca2+]i is likely due to a slowing of the Ca2+-H+ exchange mechanism in the acidic organelles that we and others have previously identified in human platelets [5, 12]. The effect of Cyt D would appear to be through eliciting an enhanced H+ sequestration into the acidic organelles, as this actin polymerisation inhibitor still slowed alkalinisation of the acidic organelles even when the H+-permeability of the granules was significantly enhanced by treatment with the K+/H+ antiporter, nigericin. Although a reduction in H+ release from the granules cannot be fully ruled out, it would be expected that the high concentration of nigericin used in this study would compensate for any minor changes in organellar H+ permeability by fully permeabilising the internal membrane to H+ ions.

The mechanism by which Cyt D enhances H+ sequestration into the acidic organelles is unclear. Platelets are known to possess a v-H+-ATPase which is required for Ca2+ sequestration into acidic organelles [5, 9, 28]. Therefore, it is possible that Cyt D treatment enhances the activity of this ATPase. Previous studies have demonstrated that the v-H+-ATPase possesses actin binding sites [29 – 31] which may play a role in regulating its activity or its localisation within the cell. Additionally, previous work in other cell types has demonstrated that actin cytoskeletal remodelling alters H+-ATPase numbers internally by regulating the recycling of this transporter between the plasma membrane and intracellular stores [32, 33]. By enhancing v-H+-ATPase activity during ADP stimulation, there is reduced alkalinisation of the acidic organelle ensuring the pH gradient across this membrane is maintained. This allows Ca2+-H+ exchange to work at near-maximal levels throughout the ADP-evoked Ca2+ signal in Cyt D treated cells. In contrast, in control cells, the alkalinisation of the acidic organelle significantly reduces this pH gradient, blunting Ca2+-H+ exchange at later times points, allowing for the formation of the secondary plateau in [Ca2+]i observed in ADP-stimulated platelets.

These data provide the first evidence that the pH gradient across acidic organelles can be dynamically regulated upon human platelet activation, and that this can play a significant role in controlling human platelet function by modulating Ca2+-H+ exchange. The data presented here also indicate that autocrine 5-HT signalling potentiates the ADP-evoked cytosolic Ca2+ signal principally by regulating the pH gradient across acidic organelle membranes, as inhibiting or enhancing the autocrine 5-HT signal results in corresponding changes in the ADP-evoked alkalinisation of the acidic organelles. The mechanism by which 5-HT can trigger this response is currently unclear but may involve opening of H+-permeable ion channels in the acidic organelle membrane, additional regulation of the activity of the v-H+-ATPase or effects on other H+ exchange mechanisms across acidic organelle membranes. Further work will be required to assess how autocrine 5-HT elicits this alkalinisation. Interestingly Cyt D appears to reduce the effectiveness of 5-HT signalling between the plasma membrane and the acidic organelles, as the 5-HT secretion data shows that the effect of Cyt D is to enhance the amount of extracellular 5-HT available for signalling after ADP stimulation, but that this 5-HT is less effective at causing changes in acidic organelle pH. This is likely due to the previously described ability of Cyt D treatment to counter the alkalinisation of the acidic organelle through enhancing resequestration of H+. This effect would therefore require significantly greater autocrine 5-HT signalling to induce the same level of alkalinisation, to induce the same inhibitory effect on the Ca2+/H+ exchange mechanism, This effect is shown in the graphical abstract. In the presence of fluoxetine, the supraphysiological extracellular concentration of 5-HT induced after ADP stimulation elicits a drop in the acidic organelle pH below a threshold level that equally prevents Ca2+/H+ exchange from occuring in both Cyt D- and DMSO-treated cells. This normalises the cytosolic Ca2+ response, and thus overcomes the inhibitory effect of Cyt D by inducing significant alkalinisation even in the presence of Cyt D.

Currently the molecular identity of the Ca2+-H+ exchange mechanism in human platelets is unknown. As this pathway persists even in the presence of high concentrations of thapsigargin and TBHQ, it appears unlikely to represent SERCA3 activity. In lysosome-related organelles in other cell types Ca2+-H+ exchange mechanisms are thought to be due to the activity of two or more coupled transporters. For example, in melanosomes a v-H+-ATPase provides a proton gradient to drive a Na+-H+ exchanger, and the resulting Na+ gradient then drives the K+-dependent Na+-Ca2+ exchanger NCKX5 to transport Ca2+ into the melanosome [34].

*In vivo* studies have demonstrated that prolonged Ca2+ signals are important in ensuring thrombus growth by preventing platelets from detaching from developing thrombi [35] and prolonged elevations in [Ca2+]i are important in mediating platelet transition into a procoagulant phenotype [36]. Thus, mechanisms that prolong Ca2+ signals are key mediators of thrombus growth and enhanced local fibrin deposition. Hence mechanisms that prolong platelet Ca2+ signals could contribute to arterial thrombosis by encouraging excessive clot formation. The work presented here demonstrates that regulation of Ca2+ sequestration by acidic organelles could impact upon the extent of blood clot formation. Previous data has demonstrated that enhanced 5-HT signalling is associated with a higher risk of cardiovascular events [37]. The work presented here suggests one mechanism by which this might occur.

The functional importance of platelet acidic Ca2+ stores is also indicated by work with SERCA3 knockout mice. Knockout of SERCA3, which contributes to acidic store Ca2+ filling, has been shown to reduce autocrine ADP secretion and so result in defective thrombus formation and platelet aggregation [38, 39]. These studies suggest Ca2+ release from acidic stores may lead to secretion of a specific population of ADP-containing dense granules, with this autocrine ADP being important in responses to weak stimuli. A role for autocrine 5-HT in Ca2+ signalling has also been demonstrated in murine platelets, where elimination of 5-HT stores in platelets after knockout of the 5-HT transporter, SERT, resulted in reduced store operated Ca2+ entry [40].

In summary, we present here evidence that autocrine 5-HT signalling plays an important role in regulating acidic store Ca2+ sequestration in human platelets by a mechanism involving alteration of the pH gradient between the acidic store lumen and the cytosol. These data provide new insight into how autocrine 5-HT can play a non-redundant role in maintaining cytosolic Ca2+ signals alongside other constituents of the platelet dense granules. The signalling pathways that link extracellular 5-HT signalling to the acidic Ca2+ stores are regulated by the actin cytoskeleton. This demonstrates for the first time that Ca2+-H+ exchange into the acidic organelles is actively modulated by intracellular signalling pathways and that this mechanism plays a role in shaping agonist-evoked Ca2+ signals.

**Declaration of Competing Interests**

The authors have no conflicts of interest to declare

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**Figure Legends**

**Figure 1. The secondary phase of ADP-evoked rise in [Ca2+]i is due to reduced autocrine 5-HT signalling.** (A, B)Fura-2-loaded platelets suspended in supplemented HBS were incubated with 10 µM Cyt D (10 µM) or its vehicle, DMSO, for 40 min at 37ºC, or KB-R7943 (50 µM) was added 2 min before the cells were stimulated. Fluoxetine (20 µM) (B) or its vehicle, DMSO, (A) and EGTA (final concentration 1 mM) were added immediately before the start of recording. 15 s later the cells were stimulated by addition of ADP (50 µM).

**Figure 2. Disruption of the actin cytoskeleton using cytochalasin D or inhibiting dense granule secretion using KB-R7943 abolishes a secondary phase of ADP-evoked rise in [Ca2+]i in the absence of extracellular Ca2+.** Fura-2-loaded platelets suspended in supplemented HBS were incubated with (A) 10 µM Cyt D (10 µM) or its vehicle, DMSO, for 40 min at 37ºC or (B) KB-R7943 (50 µM) or its vehicle, DMSO, were added 2 min before the cells were stimulated. (A, B) 30 s before the start of recording EGTA (final concentration 1 mM) was added to chelate extracellular Ca2+. 20 s after the start of recording the cells were stimulated by addition of ADP (50 µM).

**Figure 3. The effect of Cyt D on the secondary phase of ADP-evoked rise in [Ca2+]i is not due to reduced secretion or enhanced reuptake of 5-HT.** Platelets were loaded with 3H-5HT as described in Materials and Methods and some cells were incubated with 10 µM Cyt D (10 µM) or its vehicle, DMSO, for 40 min during the 3H-5HT loading period. Fluoxetine (20 µM), or its vehicle DMSO were then added before the cells were stimulated with ADP (50µM) or its vehicle, HBS, was added.

**Figure 4. The secondary phase of the ADP-evoked rise in [Ca2+]i is due to reduced Ca2+ sequestration into acidic organelles which is dependent on the actin cytoskeleton.** Fura-2- (A) or Fluo-5N-loaded platelets (B, C) suspended in supplemented HBS were incubated with 10 µM Cyt D (10 µM) or its vehicle, DMSO, for 40 min at 37ºC. 15 s after the start of recording EGTA (final concentration 1 mM) was added to chelate extracellular Ca2+ and 15 s later the cells were stimulated with ADP (50µM) and simultaneously nigericin (Nig; 10 µM) (A, C) or its vehicle, ethanol (EtOH) (A, B) were added.

**Figure 5. ADP evoked acidic organelle alkalinisation and cytosolic acidification are altered after Cyt D treatment.** Lysosensor Green DND-189- (A, B) or BCECF-loaded platelets (C, D) suspended in supplemented HBS were incubated with 10 µM Cyt D (10 µM) or its vehicle, DMSO, for 40 min at 37ºC. 15 s after the start of recording EGTA (final concentration 1 mM) was added to chelate extracellular Ca2+ and 15 s later the cells were stimulated with ADP (50µM) and (B, D) nigericin (10 µM) or (A, C) its vehicle, EtOH, were added simultaneously.

**Figure 6. Modulators of 5-HT signalling affect ADP-evoked changes in [Ca2+]st, pHao and pHi.** Fluo-5N- (A, B), Lysosensor Green DND-189- (C,D) or BCECF-loaded platelets (E, F) were suspended in supplemented HBS. Fluoxetine (20 µM) was added immediately before (A-C) or ketanserin (50 µM) was added 2 min before (D-F) the start of experiments. 30 s after the start of recording EGTA (final concentration 1 mM) was added to chelate extracellular Ca2+ and 30 s later the cells were stimulated by the addition of ADP (50µM).

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**Figure 1**

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**Figure 2**

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**Figure 6**

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**Graphical Abstract**