**Human platelets use a cytosolic Ca2+ nanodomain to activate Ca2+-dependent shape change independently of platelet aggregation**

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**Abstract**

Human platelets use a rise in cytosolic Ca2+ concentration to activate all stages of thrombus formation, however, how they are able to decode cytosolic Ca2+ signals to trigger each of these independently is unknown. Other cells create local Ca2+ signals to activate Ca2+-sensitive effectors specifically localised to these subcellular regions. However, no previous study has demonstrated that agonist-stimulated human platelets can generate a local cytosolic Ca2+ signal. Platelets possess a structure called the membrane complex (MC) where the main intracellular calcium store, the dense tubular system (DTS), is coupled tightly to an invaginated portion of the plasma membrane called the open canalicular system (OCS). Here we hypothesised that human platelets use a Ca2+ nanodomain created within the MC to control the earliest phases of platelet activation. Dimethyl-BAPTA-loaded human platelets were stimulated with thrombin in the absence of extracellular Ca2+ to isolate a cytosolic Ca2+ nanodomain created by Ca2+ release from the DTS. In the absence of any detectable rise in global cytosolic Ca2+ concentration, thrombin stimulation triggered Na+/Ca2+ exchanger (NCX)-dependent Ca2+ removal into the extracellular space, as well as Ca2+-dependent shape change in the absence of platelet aggregation. The NCX-mediated Ca2+ removal was dependent on the normal localisation of the DTS, and immunofluorescent staining of NCX3 demonstrated its localisation to the OCS, consistent with this Ca2+ nanodomain being formed within the MC. These results demonstrated that human platelets possess a functional Ca2+ nanodomain contained within the MC that can control shape change independently of platelet aggregation.

**Keywords**: Calcium, nanodomain, human platelet, membrane complex, membrane contact site, shape change

**Abbreviations:** [Ca2+]cyt - cytosolic Ca2+ concentration; DM-BAPTA - 5-5’-dimethyl-BAPTA; DTS - Dense Tubular System; IP3R - IP3 Receptor; IP3R1/2 – Type 1/2 IP3 Receptor; MC - membrane complex; MCS – Membrane Contact Site; MLCK – Myosin light chain kinase; [Na+]cyt – cytosolic Na+ concentration; NCX - Na+/Ca2+ exchanger; OCS - Open Canalicular System; PM – Plasma Membrane

1. **Introduction**

Being able to control the spread of Ca2+ through the cytosol to generate specific microdomains is fundamental to cells ability to use Ca2+ signals as a second messenger system. By localizing specific Ca2+-dependent effectors to discrete microdomains in different subcellular regions, cells can use this divalent cation to independently control a range of different functions in the cells. [1,2]. Human platelets use a rise in cytosolic Ca2+ concentration ([Ca2+]cyt) to trigger all stages of thrombus formation, including shape change, adhesion, granule secretion, aggregation, induction of pro-coagulant activity and clot retraction [3]. Human platelets need to selectively activate these different Ca2+-dependent functions as the thrombus develops to allow its orderly formation. However how platelets are able to use Ca2+ signalling to regulate this currently remains unclear. The problem lies in the tiny size of the resting platelet (2-3µm diameter), as this corresponds to the spatial spread of the Ca2+ microdomains seen in other cells [4]. Thus, the generation of a Ca2+ microdomain in a platelet would trigger a rise in [Ca2+]cyt right across the cell, triggering the activation of all the different Ca2+-sensitive effectors simultaneously. This is consistent with imaging of cytosolic Ca2+ signals in single agonist-stimulated human platelets, where only global cytosolic Ca2+ signals that spread across the cell are observed [5-6]. This inability to create local Ca2+ signals in discrete subcellular regions would seemingly preclude human platelets from being able to use local Ca2+ signals to independently control the different stages of their activation.

*Membrane contact sites* (MCS)are formed by the tight apposition of two distinct membranes (within 10-30 nm of one another) which create an isolated cytosolic nanodomain in which Ca2+ diffusion is restricted [7]. These structures therefore provide a cellular architecture capable of creating highly-localised Ca2+ signals that can be used to selectively contain Ca2+ spread through the cell, thus creating Ca2+ nanodomains independently of Ca2+ rises in the bulk cytosol. These spaces can allow selective control of downstream Ca2+-sensitive signalling pathways through co-localisation of Ca2+ effectors into the cytosolic nanodomain contained with the MCS [7,8]. However, due to their miniscule volume and the rapid kinetics of Ca2+ signals formed in these spaces, these cytosolic Ca2+ nanodomains are beyond the spatiotemporal resolution of conventional fluorescence microscopy [9-11]. The presence of such “silent” cytosolic Ca2+ signals has been previously demonstrated at the membrane contact sites of a number of other cell types [7,10, 12-15]. These undetectable Ca2+ signals are instead indirectly observed either through monitoring their ability to refill intracellular Ca2+ stores [7, 12-14] or by monitoring Ca2+ removal from these nanodomains into the extracellular space [10, 15].

Human platelets possess a MCS called the membrane complex (MC) [16,17]. This structure is formed by the intimate apposition of an invaginated portion of the plasma membrane (PM) called the open canalicular system (OCS) and the platelet equivalent of the smooth endoplasmic reticulum called the dense tubular system (DTS). Previously, we provided evidence that IP3 receptor (IP3R)-mediated Ca2+ release must be coupled to Na+/Ca2+ exchanger (NCX)-mediated Ca2+ removal at the MC [18,19] Therefore, we hypothesised that the cytosolic space within the MC might generate a Ca2+ nanodomain in human platelets that can selectively control specific changes in platelet function.

5-5’-dimethyl-BAPTA (DM-BAPTA) has been widely used to test for the presence of Ca2+ nanodomains in other cells [11,20-21]. Due to its high affinity and fast Ca2+ binding kinetics, this chelator is able to effectively buffer Ca2+ rises in the bulk cytosol but cannot buffer within 30 nm of an open Ca2+ channel [22-25]. Previously published images have shown that the membranes of the OCS and DTS lie within around 10-20 nm of each other [16,17]. Thus if the IP3R and NCX are coupled at the MC, DM-BAPTA loading should not prevent Ca2+ released from the DTS from entering the extracellular fluid via NCX-mediated transport across the plasma membrane in the OCS. This would allow NCX-mediated Ca2+ removal from the Ca2+ nanodomain to be used as an indirect measure of free Ca2+ present in this subcellular domain. Here we use this strategy to attempt to isolate a Ca2+ nanodomain in human platelets, and assess whether Ca2+ signals in this region can trigger Ca2+-dependent shape change in the absence of a detectable Ca2+ rise in the remainder of the cytosol.

1. **Materials and Methods**

**2.1 Materials**

Fura-2/AM and SBFI/AM were from Cambridge Biosciences (Cambridge, UK). Thrombin was from Merck Chemicals (Nottingham, UK). Fluo-4 K+ salt was from Invitrogen (Paisley, UK). KB-R7943, Nicergoline and Y-27632 were from Tocris Bioscience (Bristol, UK). 2-APB, Apyrase, 5-5′-Dimethyl-BAPTA/AM, 2-APB, ML-7 and Poly-L-Lysine solution were from Sigma Aldrich (Gillingham, UK). Goat NCX3 (C-15) antibody was from Insight Biotechnology (Wembley, UK). Preabsorbed Alexa Fluor555-conjugated donkey anti-goat antibody was from Abcam (Cambridge, UK), Sheep anti-mouse IgG HRP conjugated antibody was from GE Healthcare (little Chalfont, UK). Nunc 8-well chambered cover slides were obtained from R+D systems (Rochester, NY). All other reagents were of analytical grade.

**2.2 Human Platelet Preparation**

This study was approved by the Keele University Research Ethics Committee. Healthy, drug free volunteers donated blood after giving written informed consent. Blood was collected via venepuncture and mixed with one-sixth volume of acid citrate dextrose anticoagulant (ACD; 85 mM sodium citrate, 78 mM citric acid, and 111 mM glucose). This blood was then subjected to centrifugation for 8 min at 700g. Platelet rich plasma (PRP) was then collected from above the buffy coat and immediately treated with 100 μM aspirin and 0.1 U/mL apyrase.

**2.3 Monitoring thrombin-evoked rises in cytosolic and extracellular Ca2+ concentrations, and cytosolic Na+ concentration in washed human platelet suspensions**

Thrombin-evoked changes in [Ca2+]cyt or cytosolic Na+ concentration ([Na+]cyt)were monitored in Fura-2- or SBFI-loaded platelets respectively, using our previously published methodologies [18]. Changes in extracellular Ca2+ concentration ([Ca2+]ext) were monitored using washed platelets resuspended in the presence of 2.5 μM Fluo-4 K+ salt.11 Experiments were performed on 1.2 mL magnetically-stirred aliquots of washed human platelet suspensions held at 37°C. Fluorescent measurements were recorded using a Cairn Research Spectrophotometer (Cairn Research, Faversham, UK). [Ca2+]cyt, [Na+]cyt and [Ca2+]ext, were quantified by integration of the change in fluorescence records from basal with respect to time for 3.5 min after thrombin addition.

**2.4 Imaging of thrombin-evoked changes in [Ca2+]ext in single human platelets**

Ca2+removal into the pericellular region of individual platelets was monitored in washed platelet suspensions to which Fluo-4 K+ salt had been added [18]. Washed platelets were collected by centrifugation of PRP at 350 *g* for 20 min. Washed platelets were resuspended at a density of 2x108 cells.mL-1 in Hepes-buffered saline (HBS; 145 mM NaCl, 10 mM Hepes (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid), 5mM KCl, 1 mM MgSO4, pH 7.45) supplemented on the day with 10 mmol.L−1 d-glucose, 0.1% [w/v] bovine serum albumin, 200 μmol.L−1 CaCl2 and 0.1 U.mL−1 apyrase (supplemented HBS). Washed platelets were incubated with 30 μM DM-BAPTA/AM for 10 minutes at 37°C under continuous magnetic stirring. 50 μM KB-R7943 or an equivalent volume of its vehicle, DMSO added for the last 5 min of this incubation. Cells were then allowed to settle for 5 min at room temperature on poly-L-lysine-coated Nunc chambered cover slides. Excess cell suspension was removed and replaced with supplemented HBS to which 1 mM EGTA and 5 μM Fluo-4 K+ salt was added. Transmitted light and fluorescent images were recorded using a Fluoview FV 1200 laser scanning confocal microscope (Olympus, UK) with a 60x oil immersion objective using a 100-μm confocal aperture. Fluorescence images were recorded at a frequency of 0.5 Hz for 5 min using an excitation wavelength of 473 nm and collecting emitted light between 520-560 nm.

**2.5 Immunofluorescent imaging of NCX3 in single platelets**

Unstimulated, washed human platelet suspensions were fixed by addition of 3% [w/v] formaldehyde and stored at 4°C until use. Fixed platelets were washed by centrifugation at 6000 *g* for 1 min and resuspended in HBS containing a 1:1000 dilution of Horseradish Peroxidase-conjugated Sheep anti-mouse secondary antibody and 5 mg.mL-1 BSA and incubated for 30 min at room temperature to block non-specific antibody binding sites. Cells were recollected by centrifugation and platelets resuspended in HBS containing a 1:25 dilution of goat anti-NCX3 antibody and 5mg.mL-1 BSA. Cells were incubated with antibodies at room temperature for 30 min and then recollected by centrifugation and resuspended in HBS containing a 1:1000 dilution of pre-absorbed Alexa Fluor 555-conjugated Donkey anti-goat antibody and 5mg.mL-1 BSA. The platelets were incubated with this fluorophore-conjugated secondary antibody at room temperature for 30 min. Cells were recollected one last time by centrifugation and resuspended in HBS containing 5 mg.mL-1 BSA. Labelled cells were loaded onto poly-L-lysine-coated Nunc Chambered cover slides and allowed to adhere to the surface for 10 min at room temperature. Platelet suspension was then removed and slides were washed with HBS containing 5 mg.mL-1 BSA. Fluorescent images were captured using a Fluoview FV1200 laser scanning confocal microscope (Olympus, UK) with a PLAPON 60x oil immersion objective. Images were recorded at a frequency of 0.5 Hz for 5 min with excitation at 473 nm and emission light at 520-560 nm.

**2.6 Monitoring platelet shape change using light transmission aggregometry**

Washed human platelets were collected by centrifugation of PRP at 350 *g* for 20 min and resuspended in supplemented HBS. Cells were pre incubated with their assigned treatments under magnetic stirring at 37°C. External Ca2+ was then chelated by addition of 1 mM EGTA and 450 μL of suspension was then transferred into aggregometer cuvettes (Kartell, Italy). Readings were taken under continuous magnetic stirring at 37°C using a 2-channel Chronolog light transmission aggregometer (Labmedics, Oxfordshire, UK). Readings were made against a baseline level of an aggregometer cuvette containing supplemented HBS.

**2.7 Statistical analysis**

Results are reported as the mean ± SEM of the number of independent observations (n) made. Statistical significance was assessed using either Student’s paired t-test or a one-way ANOVA test followed by a *post hoc* Tukey test. *P* < 0.05 was considered significant.

1. **Results**

**3.1 NCX-mediated Ca2+ removal is coupled to Ca2+ release from the dense tubular system at a membrane contact site**

Initial experiments were performed to assess whether IP3R-mediated Ca2+ release from the DTS is coupled to NCX-mediated Ca2+ removal at a membrane contact site. Platelets were loaded with DM-BAPTA at concentrations previously demonstrated to block rises in the bulk cytosolic Ca2+ concentration ([Ca2+]cyt). DM-BAPTA-loaded platelets were stimulated with thrombin in a Ca2+-free, EGTA-chelated HBS to ensure Ca2+ release from the platelet intracellular Ca2+ stores was the sole source of this divalent cation [18]. This preparation ensures the only available Ca2+ in these cells will be in the nanodomain surrounding open Ca2+ release channels (Fig. 1) [22-25]. If IP3R and NCX are localised at a membrane contact site then thrombin-evoked rises in the extracellular Ca2+ concentration should be observable in DM-BAPTA loaded cells.

Positive control experiments performed on each donor demonstrated that pre-incubation of Fura-2-loaded human platelets with DM-BAPTA was able to abolish thrombin-evoked rises in [Ca2+]cyt (1.0 ± 0.6 % of control; n = 4; P < 0.05; Fig. 2A), consistent with this treatment preventing rises in the bulk cytosol. Despite the lack of a measurable rise in bulk cytosolic Ca2+ concentration, Ca2+ removal into the extracellular fluid could still be observed in DM-BAPTA-loaded platelets prepared from the same blood samples, demonstrating that there is still Ca2+ available to platelet Ca2+ removal mechanisms in the cytosol that is not buffered by DM-BAPTA or detected by Fura-2. This demonstrates that Ca2+ release from intracellular stores occurs into a cytosolic nanodomain formed by the close apposition of the dense tubular system and the platelet plasma membrane.

The Ca2+ removal into the extracellular fluid from DM-BAPTA-loaded cells was observed to be slower than in control cells (69.7% ± 10.5% of untreated controls; n = 7; P < 0.05; Fig 2.B,D). This is consistent with our previously-published finding that DM-BAPTA-loading slows thrombin-evoked Ca2+ release from the intracellular Ca2+ stores [18]. This effect is due to the chelator preventing Ca2+-induced Ca2+ release from the DTS by preventing Ca2+ rises in the bulk cytosolic space, which have been previously demonstrated to accelerate Ca2+ release in human platelets[18,26,27].

To assess if the NCX was responsible for mediating this Ca2+ removal from the nanodomain, experiments were performed on platelet samples from the same donors to examine if the NCX inhibitor, KB-R7943, could reduce this Ca2+ removal. We have previously demonstrated that this compound inhibits forward-mode NCX activity in human platelets, whilst not directly blocking Ca2+-permeable ion channels in the plasma membrane or intracellular stores [18,28]. Pre-treatment with KB-R7943 significantly reduced the thrombin-evoked rises in [Ca2+]ext both in control cells (37.5% ± 3.3% of control; n = 7, P < 0.05, Fig 2D) as well as those loaded with DM-BAPTA (35.4% ± 7.7% of control; n = 7; P < 0.05; Fig 2E). Pretreatment also reduced the baseline fluo-4 fluorescence seen prior to stimulation with thrombin (86.5% ± 3.3% of control; P < 0.05). This difference is likely due to KB-R7943 preventing NCX3-mediated removal of Ca2+ into the extracellular fluid during the preincubation period. These results suggest that the NCX plays a role in preventing cytosolic Ca2+ rises even in resting platelets. Normalisation of the data relative to the baseline fluorescence prior to thrombin stimulation did not alter the results observed (See Figure S1 and Table S1 in supplementary materials).

If the NCX was responsible for the Ca2+ removal observed in DM-BAPTA-loaded cells, a KB-R7943-sensitive rise in cytosolic Na+ concentration ([Na+]cyt) would also be observable upon thrombin stimulation due to the influx of Na+ on this exchanger. As shown in Fig 2F, a clear thrombin-evoked rise in [Na+]cyt was observed in DM-BAPTA loaded cells, which was significantly reduced by KB-R7943-pretreatment (21.6 ± 7.5% of control; *n* = 6; *P* < 0.05), consistent with the role of the NCX in eliciting the observed Ca2+ removal from the Ca2+ nanodomain. Baseline SBFI fluorescence observed immediately prior to thrombin stimulation was also significantly reduced in KB-R7943-treated cells (82.9% ± 2.2% of control; P < 0.05), consistent with NCX-mediated Ca2+ removal being continually active, even in resting platelets. The Na+ entering through the NCX must therefore provide a contribution to the steady-state cytosolic Na+ concentration observed in resting platelets. Normalisation of this data did not alter the results observed (Figure S2 and Table S1 in supplementary materials).

**3.2 Nicergoline-mediated disruption of the subcellular localisation of the DTS inhibits Ca2+ removal from the cytosolic nanodomain**

To confirm that the DTS is the intracellular Ca2+ store responsible for supplying Ca2+ to the NCX, we assessed how disrupting the normal subcellular localisation of the DTS in the cell affected NCX-mediated Ca2+ removal in DM-BAPTA-loaded cells. Previous studies have demonstrated that nicergoline treatment is able to disrupt the normal localisation of the DTS [19,29]. Experiments were performed to examine if nicergoline-induced redistribution of the DTS was able to disrupt thrombin-evoked efflux of Ca2+ from the cytosolic nanodomain. Nicergoline pre-treatment of DM-BAPTA-loaded platelets significantly inhibited thrombin-evoked rises in [Ca2+]ext in cells from the same donors to 51.0 ± 19.6% of control (n = 9; *P* < 0.05; Fig 3A,B). Nicergoline pretreatment also significantly reduced thrombin-evoked rises in [Na+]cyt in DM-BAPTA-loaded platelets to 23.6 ± 5.4% of control (n = 6; *P* < 0.05; Fig 3C). Positive control experiments confirmed that DM-BAPTA-loading could prevent observable Ca2+ rises in the bulk cytosol (3.8 ± 1.6% of control; n = 4; *P* < 0.05; Fig 3D). These results show that NCX-mediated Ca2+ removal from the DM-BAPTA-insensitive cytosolic nanodomain is reliant on the normal positioning of the DTS in human platelets. These results therefore support the hypothesis that the cytosolic nanodomain is enclosed by a close apposition of the DTS with the plasma membrane. These results are consistent with the cytosolic nanodomain being present within the MC, consistent with our previous prediction [18,19].

**3.3 DM-BAPTA-loading does not prevent the production of pericellular Ca2+ hotspots generated by NCX-mediated Ca2+ removal in single thrombin-stimulated human platelets**

It is not possible to directly image cytosolic Ca2+ nanodomains enclosed within membrane contact sites using freely-diffusible Ca2+ indicators [9-11]. This is due both to limitations of the spatiotemporal resolution of current real-time imaging techniques, as well as the diffusion of Ca2+-bound fluorescent indicators leading to the elicited fluorescence being artifactually observed over a much wider area than the Ca2+ nanodomain [9-11]. Previous studies have therefore utilised indirect methods, such as monitoring Ca2+ removal from these sites to monitor Ca2+ rises within these cytosolic spaces [15]. Previously we have demonstrated that we can observe Ca2+ removal from thrombin-stimulated platelets, and that this appears to occur into a specific pericellular hotspot. We predicted this hotspot was localised within the OCS lumen, consistent with this being generated at the MC[18,19]. If the MC is responsible for generating pericellular hotspots, then DM-BAPTA buffering of the bulk cytosol will not affect their generation. Examination of control cells showed that pericellular Ca2+ hotspots could be observed in most of thrombin-stimulated, DM-BAPTA-loaded cells during the recording period (66.1 ± 5.8% of cells per field; n = 7; *P* < 0.05; Fig 4). They were found in a region just inside the surface membrane, consistent with their localisation within the OCS. When DM-BAPTA-loaded cells were pre-incubated with KB-R7943 prior to thrombin stimulation, similar pericellular Ca2+ hotspots were still occasionally observed, although they occurred less frequently with the proportion of cells showing a hotspot being significantly reduced to 39.7 ± 3.1% of cells per field (n = 10; P < 0.05). These data therefore demonstrate that the pericellular Ca2+ hotspots are generated by NCX-mediated transport from the Ca2+ nanodomain identified previously.

**3.4 NCX3 is localised in a specific platelet microdomain, consistent with its localisation at the MC**

NCX3 expression has been previously demonstrated to be present in human platelets [30-32], however no data currently exists for its subcellular localization in these cells. Previously we demonstrated the presence of NCX3 using a primary antibody raised to an extracellular epitope of this exchanger [30]. Using this antibody we have performed immunofluorescent labelling of NCX3 in intact platelets to assess whether this transporter is found in the OCS. Fluorescent imaging of DMSO-treated platelets found that cells possessed a punctate distribution of NCX3 protein predominantly with 1 or 2 main puncta just beneath (but not on) the platelet surface membrane, consistent with its presence in the open canalicular system (Fig 5). These puncta were observed in 92% of all cells labelled with the NCX3 antibody. The remaining cells either showed no labelling (6%) or widespread fluorescence throughout the cell (2%; n = 50 cells observed over 4 different donors). Cells incubated with the secondary antibody alone showed limited labelling with 82% of cells showing no fluorescent labelling. 15% of the cells could be seen to have weak fluorescent signals in part of the cell, and 3% showed widely-distributed fluorescence through the whole cell (n = 60 cells from the same 4 donors). These data indicate that non-specific binding of the secondary antibody alone to our fixed platelets is uncommon, cannot account for the fluorescent labelling seen in our cells. The punctate distribution of NCX3 is consistent with its presence at the MC, which is generally found in 1 or 2 eccentric locations just inside the platelet surface membrane [16,17]. This distribution is also consistent with the location of thrombin-evoked pericellular Ca2+ hotspots (Fig. 4)[18,19]. These results support the possibility that NCX3 is specifically localised to the portion of the OCS membrane that forms the MC with the DTS.

**3.5 Platelet shape change is triggered by rises in Ca2+ concentration within the cytosolic nanodomain**

Having identified a silent Ca2+ nanodomain in human platelets, we next considered whether this localised Ca2+ signal could be responsible for triggering changes in platelet function. As Ca2+ release from the DTS is the first source of Ca2+ upon stimulation with most platelet agonists [33], we predicted that the Ca2+ nanodomain would be responsible for triggering early functional changes in human platelets. As shape change occurs rapidly after human platelet activation, we hypothesised that Ca2+ rises in the nanodomain in response to Ca2+ release would be able to trigger this response in isolation from Ca2+ rises in the bulk cytosol. Previous studies have concluded that platelet shape change consists of both Ca2+-dependent and Ca2+-independent pathways based on their sensitivity to BAPTA loading, with the Ca2+-dependent pathway being effected by myosin light chain kinase (MLCK), whilst the Ca2+-independent pathway required the activity of Rho Kinase [34]. Light transmission aggregometry was used to examine whether the BAPTA-insensitive platelet shape change was triggered by Ca2+ rises within the identified NCX-associated cytosolic nanodomain. Blocking the NCX using KB-R7943 will reduce Ca2+ removal from the cytosolic nanodomain and would therefore be predicted to potentiate shape change in DM-BAPTA-loaded platelets. To assess the Ca2+ dependence of an any observed changes platelets were also pre-treated with an inhibitor of MLCK (ML-7; to inhibit the Ca2+-dependent pathway) or Rho kinase (Y27632 to inhibit the Ca2+-independent pathway).

As KB-R7943 has limited solubility when added directly to solutions, KB-R7943 (or an equivalent volume of its vehicle, DMSO) was solubilised at high concentrations into HBS by magnetic stirring at 37°C. This pre-solubilised KB-R7943 was then added to the washed platelet suspension to ensure that KB-R7943 did not come out of solution upon addition and artificially reduce light transmittance. The addition of these drug- or DMSO- containing solutions to the platelet suspensions dilutes the platelet suspension, causing an initial increase in light transmittance (shown as a drop in the trace) in all experiments. No further change in light transmittance was then seen in platelets treated with DMSO-containing HBS (decrease in light transmittance = -0.0% ± 0.2% of basal; n = 9; Fig 6A,B). However, when platelets were exposed to 25 µM (Fig 6A) or 50 µM KB-R7943 (Fig 6B), there was a gradual, dose-dependent decrease in light transmittance. This is consistent with KB-R7943 initiating a shape change (decrease in light transmittance = 5.6% ± 0.3% and 8.5% ± 0.3% of basal for 25 µM and 50 µM KB-R7943 respectively; n = 9; both *P* < 0.05 compared to DMSO control; Fig 6C). These results suggest that the NCX may function in resting cells to ensure Ca2+ release caused by stochastic opening of IP3R channels can be rapidly removed without triggering shape change. When this pathway is blocked, Ca2+ leaks into the MC nanodomain and slowly triggers shape change. This is consistent with our previous findings that baseline cytosolic Na+ and Ca2+ concentration are reduced in KB-R7943-treated cells (Section 3.1).

After the pre-incubation period with KB-R7943 or DMSO, platelets were subsequently stimulated with thrombin to assess the impact on agonist-evoked Ca2+ signalling. As shown in Fig 6A,B, thrombin induced a rapid decrease in light transmittance consistent with the onset of shape change in DMSO-treated platelets, which then slowly returned towards baseline over the course of the observation period. In cells treated with KB-R7943 there was a further decrease in light transmittance over the KB-R7943-induced shape change, which then reversed only slightly towards baseline levels during the observation period. The maintained shape change at the end of the recording period was found to be significantly higher in KB-R7943-treated cells compared to DMSO-treated controls (Fig 6D). No platelet aggregation was observed during the recording period, consistent with previous studies [34]. Thus NCX-mediated Ca2+ removal from the cytosolic nanodomain limits the duration of thrombin-evoked shape change in DM-BAPTA-loaded platelets by limiting the duration of Ca2+ rises here. This shape change occurred without subsequent platelet aggregation, demonstrating that the Ca2+ nanodomain can trigger platelet shape change in isolation of platelet aggregation.

To assess whether the shape changes observed both by KB-R7943 treatment and thrombin stimulation in DM-BAPTA-loaded platelets were Ca2+-dependent, further experiments were performed to assess how blocking myosin light chain kinase (MLCK; Ca2+-dependent shape change) and Rho kinase (Ca2+-independent shape change) modulated these effects. As can be seen in Figure 7, pretreatment with the MLCK inhibitor, ML-7, blocked both KB-R7943- (Fig 7B,C) and thrombin-induced (Figure 7A-D) shape change in DM-BAPTA loaded platelets, suggesting that Ca2+-dependent activation of MLCK is required for both of these responses. In contrast, pre-treating platelets with the Rho Kinase inhibitor Y-27632, had no significant inhibitory effect on the KB-R7943- (Fig 7F,G) or thrombin-evoked (Fig 7E-H) shape change in DM-BAPTA-loaded platelets. These data demonstrate that both NCX inhibition and thrombin-stimulation must trigger platelet shape change through Ca2+ rises in the cytosolic nanodomain. This suggests that the rapidity of platelet shape change is caused by the rapid binding of Ca2+ to calmodulin, which can in turn swiftly trigger MLCK-dependent shape change.

**3.6 Ca2+ release from the DTS via the type 1 IP3 receptor is partly responsible for KB-R7943- and thrombin-induced shape change in DM-BAPTA-loaded platelets.**

Experiments were performed to examine whether IP3-mediated Ca2+ release from the DTS is responsible for KB-R7943 and thrombin-evoked shape change. To do this we assessed the effect of the type 1 IP3 receptor inhibitor (IP3R1), 2-APB [35] on the shape change responses observed in DM-BAPTA-loaded platelets. As shown in Figure 8, treatment with 100 µM 2-APB significantly reduced KB-R7943-induced shape change (8.0% ± 1.2% and 3.2% ± 1.0% for DMSO- and 2-APB-treated-cells respectively; n = 6; P < 0.05; Fig 8B,C). As the type 1 IP3R has been localised specifically to the DTS in human platelets [36] these results provide further evidence that the DTS is responsible for forming the cytosolic Ca2+ nanodomain. They also suggest that NCX-mediated Ca2+ removal prevents spontaneous IP3R1-mediated Ca2+ release from the DTS triggering shape change in resting platelets.

Interestingly, 2-APB did not significantly reduce the thrombin-evoked shape change in DM-BAPTA loaded platelets (9.6% ± 0.7% and 6.5% ± 1.5% in DMSO- and 2-APB-treated-cells respectively; n = 6; P = 0.09; Fig 8A), but it did significantly prolong the time taken to reach the peak response after thrombin addition (39.2 ± 1.9s and 64.6% ± 1.4s in DMSO- and 2-APB-treated-cells respectively; n = 6; P < 0.05; Fig 8D). These results suggest that blocking the type 1 IP3R slows, but does not block, thrombin-evoked Ca2+ release into the cytosolic Ca2+ nanodomain. The inability of 2-APB to fully block both thrombin- and KB-R7943-dependent shape change may be due to both IP3R1 and type 2 IP3R (IP3R2) being present in the cytosolic nanodomain, as studies have shown that both isoforms are both present in the DTS [36]. Alternatively, it is possible that Ca2+ release from the platelet acidic organelle may form a secondary Ca2+ nanodomain in a distinct subcellular region – although this is inconsistent with the findings of the imaging of the pericellular Ca2+ hotspots and localisation of NCX3. Lastly, 2-APB is not a specific inhibitor as it is also known to block SERCA activity [37], which may allow another Ca2+ leak current from the DTS to trigger shape change in the absence of NCX and SERCA function. Further study will be required to determine if Ca2+-release channels other than IP3R1 are present in the cytosolic nanodomain.

**Discussion**

A rise in cytosolic Ca2+ concentration is the principal intracellular signal that triggers all stages of human platelet activation.This has sparked significant research effort into identifying the Ca2+ channels and transporters that mediate these signals.1 However, less is known about how platelets are able to decode these signals to create functional responses. Other cell types do this through tight spatial coupling of Ca2+-permeable ion channels and Ca2+-sensitive effector molecules in specific subcellular nano- or microdomains [2,3,8,14].However, how human platelets can restrict cytosolic Ca2+ signals to allow for selective activation of effectors has remained unclear. In this paper, we utilise the known buffering properties of DM-BAPTA to isolate a previously unidentified cytosolic Ca2+ nanodomain in human platelets, providing evidence for the first such nanodomain in this cell. This signal has not been previously observed in single human platelets using freely-diffusible Ca2+ indicators such as Fura-2, Indo-1 and Fluo-4, as these indicators are unable to effectively report Ca2+ signals this small. As Ca2+-bound indicator diffuses away from the Ca2+ nanodomain, it also spreads the fluorescent signal artefactually such that the spread of the Ca2+ signal through the cytosol appears greater than it is [38]. This is particularly problematic for interpreting results in the tiny platelet, as the resting platelet is the same size as the elementary fluorescent signals observed when single ion channels are opened [4]. This means that diffusible fluorescent Ca2+ indicators report a spatially-averaged cytosolic Ca2+ signal in platelets, and will artificially mask the presence of any Ca2+ nanodomains in these cells [11]. This is consistent with previous studies which only observed global cytosolic Ca2+ signals in single human platelets monitored with diffusible Ca2+ indicators [5,6].

Here the presence of free Ca2+ in this nanodomain is indirectly observed by monitoring NCX-mediated Ca2+ removal from DM-BAPTA-loaded platelets. An analogous indirect approach has been used to monitor Ca2+ concentration in the fuzzy space of the cardiac dyad [10,15]. Whilst it is not possible to directly measure the magnitude of the cytosolic Ca2+ signal occurring within this subcellular region, it is possible to estimate that it is likely to be in the micromolar range, based upon the low-affinity nature of NCX-mediated Ca2+ transport (K0.5 0.6-6 µM) [39] and our previous demonstration that the NCX is working at around 2/3rds of its maximum speed in thrombin-stimulated platelets [18]. Interestingly, the localised rise in Ca2+ that the NCX is exposed to is not effectively reported by Fura-2 in cells co-loaded with this indicator and DM-BAPTA, demonstrating the presence of a “silent” Ca2+ signal not previously reported in human platelets. The inability to measure Ca2+ rises within nanodomains is due to both the inability of freely-diffusible Ca2+ indicators to bind Ca2+ effectively in the close vicinity of open Ca2+ channels [21-25], as well as the tiny volumes of these spaces ensuring that the number of Fura-2 molecules found diffusing through this region is likely to be insufficient to generate a detectable fluorescent signal [7-11].

The probable location for this cytosolic Ca2+ nanodomain is in the space contained within the MC. Here the OCS and DTS membranes are held within 10-20 nm of each other [16,17], thus enclosing a cytosolic nanodomain that is functionally isolated from the bulk cytosol [7]. Here we provide further evidence for this by demonstrating that disruption of the subcellular localisation of the DTS weakens NCX-mediated Ca2+ removal, suggesting that Ca2+ release from the DTS must be closely coupled to the NCX in human platelets. Additionally, NCX3 is found in a portion of the plasma membrane that is inside the surface membrane of these cells, consistent with it being present in the plasma membrane invaginations that form the OCS. Lastly, blocking the DTS-localised IP3R1 with 2-APB was able to partially inhibit the KB-R7943 shape change, and significantly slow thrombin-evoked shape change in DM-BAPTA-loaded cells. The possibility that the membrane complex is a central regulator of platelet Ca2+ signalling was suggested by mathematical modelling of thrombin-evoked platelet Ca2+ fluxes [18], as well as previous clinical case studies that have demonstrated that patients with abnormal MC morphology display platelet function defects [40-43]. This includes a case report of a family shown to have a heritable MC disorder, which leads to a defect in thrombin-evoked Ca2+ signalling [40].

Membrane complex sites, such as the MC, are known to enclose a cytosolic nanodomain with restricted Ca2+ diffusion [7], thus creating a cytosolic nanodomain that allows Ca2+ signals generated here to remain isolated from the bulk cytosol. The demonstration of subcompartmentalisation of the platelet cytosol in this way provides us with a theoretical basis for understanding how platelets are able to independently trigger downstream Ca2+-dependent functional responses. Here we demonstrate that Ca2+-dependent platelet shape change can occur without triggering Ca2+-dependent platelet aggregation. Through differentially localising Ca2+-sensitive effectors between the membrane complex and the bulk cytosol, it could be possible to selectively activate the corresponding downstream platelet responses with different Ca2+ signals. Ca2+-dependent platelet shape change is regulated by Ca2+-calmodulin activation of MLCK [38], suggesting calmodulin is present in the membrane complex. Previous studies have shown that IP3R1 possesses a Ca2+-independent calmodulin-binding domain, which may facilitate recruitment of calmodulin to this nanodomain [44]. We hypothesis that calmodulin bind to Ca2+ release into the nanodomain, which can then diffuse out of the nanodomain triggering MLCK activation and shape change.

The presence of a Ca2+ nanodomain resistant to DM-BAPTA-loading in human platelets also requires investigators to be cautious when interpreting studies that use this exogenous chelator to assess the Ca2+-dependence of platelet signalling pathways or functional responses. Whilst DM-BAPTA loading prevents Ca2+ rises in the bulk cytosol, it is unable to prevent Ca2+ rises in the cytosolic nanodomain, as observed by the continued ability of the NCX to transport Ca2+ into the extracellular space in DM-BAPTA loaded cells. Thus even with DM-BAPTA loading, it is possible that functional responses can still be triggered in a Ca2+-dependent manner through activation in this nanodomain. Therapies that selectively target the membrane complex that holds together the OCS and DTS could slow platelet activation by decoupling Ca2+ release from downstream Ca2+ effectors. Further investigation of the structure and role of the membrane complex in regulating human platelet signalling may provide novel avenues for creating anti-platelet agents.

1. **Acknowledgements**

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1. **Conflict of Interest Statement**

The authors have no conflicts of interest to declare

1. **References**
2. M.D. Bootman, P. Lipp, M.J. Berridge, The organisation and functions of local Ca2+ signals, J Cell Sci 114 (2001) 2213-2222.
3. R. Rizzuto, T. Pozzan T, Microdomains of intracellular Ca2+: molecular determinants and functional consequences, Physiol Rev 86 (2006) 369-408.
4. A.G.S. Harper, S.O. Sage, Platelet Signalling: Calcium. In: Gresele P, Kleiman NS, Lopez JA, Page CP (Eds.), Platelets in thrombotic and non-thrombotic disorders – Pathophysiology, Pharmacology and therapeutics, Cham, Springer, 2017: pp286-292.
5. E. Niggli, N. Shirokova, A guide to sparkology: the taxonomy of elementary cellular Ca2+ signalling events, Cell Calcium 42 (2007) 379-387.
6. J.W. Heemskerk, G.M. Willems, M.B. Rook, S.O. Sage, Ragged spiking of free calcium in ADP-stimulated human platelets: regulation of puff-like calcium signals in vitro and ex vivo, J Physiol 535 (2002) 625-635.
7. Y. Tsunoda, K. Matsuno, Y. Tashiro, Spatial distribution and temporal change of cytoplasmic free calcium in human platelets, Biochem Biophys Res Commun 156 (1988) 1152-1159.
8. C. van Breemen, N. Fameli, A.M. Evans, Pan-junctional sarcoplasmic reticulum in vascular smooth muscle: nanospace Ca2+ transport for site- and function-specific Ca2+ signalling, J Physiol 591 (2013) 2043-2054.
9. G.J. Kargacin, Calcium signalling in restricted diffusion spaces, Biophys J 1994;**67**: 262-272
10. L.H. Tay, I.E. Dick, W. Yang, M. Mank, O. Griesbeck, D.T. Yue, Nanodomain Ca²⁺ of Ca²⁺ channels detected by a tethered genetically encoded Ca²⁺ sensor, Nat Commun 3 (2012) 778.
11. E. Niggli, Measuring calcium in 'fuzzy' spaces, J Physiol 589 (2011) 2663.
12. M.R. Tadross, R.W. Tsien, D.T. Yue, Ca2+ channel nanodomains boost local Ca2+ amplitude, Proc Natl Acad Sci U S A. 110 (2013) 15794–15799.
13. H. Jousset, M. Frieden, N. Demaurex, STIM1 knockdown reveals that store-operated Ca2+ channels located close to sarco/endoplasmic Ca2+ ATPases (SERCA) pumps silently refill the endoplasmic reticulum, J Biol Chem 282 (2007) 11456-11464.
14. C.M.L. Di Giuro, N. Shrestha, R. Malli, K. Groschner, C. van Breemen, N. Fameli, Na +/Ca2+ exchangers and Orai channels jointly refill endoplasmic reticulum (ER) Ca2+ via ER nanojunctions in vascular endothelial cells, Pflugers Arch 469 (2017) 1287–1299.
15. O.A. Cabello, W.P. Schilling, Vectorial Ca2+ flux from the extracellular space to the endoplasmic reticulum via a restricted cytoplasmic compartment regulates inositol 1,4,5-trisphosphate-stimulated Ca2+ release from internal stores in vascular endothelial cells, Biochem J 1295 (1993) 357-366.
16. K. Acsai, G. Antoons, L. Livshitz, Y. Rudy, K.R. Sipido, Microdomain [Ca²⁺] near ryanodine receptors as reported by L-type Ca²⁺ and Na+/Ca²⁺ exchange currents, J Physiol 589 (2011) 2569–2583
17. J.G. White, Interaction of membrane systems in blood platelets, Am J Pathol 66 (1972) 295-312.
18. H. van Nispen tot Pannerden, F. de Haas, W. Geerts, G. Posthuma, S. van Dijk, H.F. Heijnen. The platelet interior revisited: electron tomography reveals tubular alpha granule subtypes. Blood 116 (2010) 1147-1156.
19. S.O. Sage, N. Pugh, R.W. Farndale, A.G.S. Harper, Pericellular Ca(2+) recycling potentiates thrombin-evoked Ca(2+) signals in human platelets, Physiol Rep 1 (2013) e00085
20. T. Walford, F.I. Musa, A.G.S. Harper, Nicergoline inhibits human platelet Ca signalling through triggering a microtubule-dependent reorganisation of the platelet ultrastructure, Br J Pharmacol 173 (2016) 234-247.
21. E. Neher, W. Almers, Fast calcium transients in rat peritoneal mast cells are not sufficient to trigger exocytosis, EMBO J 5 (1986) 51–53.
22. E. Eggermann, I. Bucurenciu, S.P. Goswami, P. Jonas, Nanodomain coupling between Ca²⁺ channels and sensors of exocytosis at fast mammalian synapses, Nat Rev Neurosci 13 (2011) 7–21.
23. M. Naraghi, E. Neher, Linearized buffered Ca2+ diffusion in microdomains and its implications for calculation of [Ca2+] at the mouth of a calcium channel, J Neurosci 17 (1997) 6961-6973.
24. E. Neher, Concentration profiles of intracellular calcium in the presence of a diffusible chelator, Exp Brain Res 14 (1986) 80–96.
25. M.D. Stern, Buffering of calcium in the vicinity of a channel pore, Cell Calcium 13 (1992) 183-192.
26. A.B. Parekh, Ca2+ microdomains near plasma membrane Ca2+ channels: impact on cell function, J Physiol 586 (2008) 3043-3054.
27. R.M. van Gorp, M.A. Feijge, W.M. Vuist, M.B. Rook, J.W. Heemskerk, Irregular spiking in free calcium concentration in single, human platelets. Regulation by modulation of the inositol trisphosphate receptors, Eur J Biochem 269 (2002) 1543-1552.
28. S.O. Sage, N. Pugh, M.J. Mason, A.G.S. Harper, Monitoring the intracellular store Ca2+ concentration in agonist-stimulated, intact human platelets by using Fluo-5N, J Thromb Haemost 9 (2011) 540-551.
29. A.G.S. Harper, M.J. Mason, S.O. Sage, A key role for dense granule secretion in potentiation of the Ca2+ signal arising from store-operated calcium entry in human platelets, Cell Calcium 45 (2009) 413-420.
30. R. Le Menn, J. Migne, R.J. Probst‐Djovakovich, Ultrastructural study on the effect of an inhibition of platelet aggregation, Arzneimittelforschung 29 (1979) 1278–1282.
31. M.T. Harper, M.J. Mason, S.O. Sage, A.G.S. Harper, Phorbol ester-evoked Ca2+ signaling in human platelets is via autocrine activation of P(2X1) receptors, not a novel non-capacitative Ca2+ entry, J Thromb Haemost 8 (2010) 1604-1613.
32. D.E. Roberts, T. Matsuda, R. Bose, Molecular and functional characterization of the human platelet Na(+) /Ca(2+) exchangers, Br J Pharmacol 165 (2012) 922–936.
33. J.M. Burkhart, M. Vaudel, S. Gambaryan et al., The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways, Blood 120 (2012) e73-e82.
34. S.O. Sage, T.J. Rink, The kinetics of changes in intracellular calcium concentration in fura-2-loaded human platelets. J Biol Chem 262 (1987): 16364-16369.
35. B.Z. Paul, J.L. Daniel, S.P. Kunapuli, Platelet shape change is mediated by both calcium-dependent and -independent signaling pathways. Role of p160 Rho associated coiled-coil-containing protein kinase in platelet shape change, J Biol Chem 274 (1999) 28293-28300.
36. H. Saleem, S.C. Tovey, T.F. Molinski, C.W. Taylor, Interactions of antagonists with subtypes of inositol 1,4,5-trisphosphate (IP3) receptor, Br J Pharmacol 171 (2014) 3298-3312.
37. T.M. Quinton, W.L. Dean, Multiple Inositol 1,4,5-Trisphosphate Receptor Isoforms are present in platelets, Biochem Biophys Res Comms 224 (1996) 740 – 746.
38. M.D. Bootman, T.J. Collins, L. Mackenzie, H.L. Roderick, M.J. Berridge, C.M. Peppiatt, 2-aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry but an inconsistent inhibitor of InsP3-induced Ca2+ release, FASEB J 16 (2002) 1145‐1150.
39. F. Sala, A. Hernandez-Cruz, Calcium diffusion modeling in a spherical neuron. Relevance of buffering properties, Biophys J 57 (1990) 313-324.
40. M.P. Blaustein, W.J. Lederer, Sodium/calcium exchange: its physiological implications, Physiol Rev 79 (1999) 763–854.
41. R.I. Parker, G.L. Bray, L.P. McKeown, J.G. White, Failure to mobilize intracellular calcium in response to thrombin in a patient with familial thrombocytopathy characterized by macrothrombocytopenia and abnormal platelet membrane complexes, J Lab Clin Med 122 (1993) 441–449.
42. D. Green, C.H. Ts'ao, I. Cohen, E.C. Rossi, Haemorrhagic thrombocytopathy associated with dilatation of the platelet—membrane complex, Br J Haematol 48 (1981) 595–600.
43. C. Canizares, N. Vivar, J. Grijalva, Thrombocytopathy due to a defect of the platelet membrane complex, Acta Haematol 83 (1990) 99–104.
44. I. Meiamed, M. Djaldetti, H. Joshua, U. Seligsohn, Association of the hemophilia A carrier state and Hemorrhagic thrombocytopathy with dilatation of the platelet membrane complex, Acta Haematol 71 (1984) 381–387.
45. N.N. Kasri, G. Bultynck, J. Smyth J et al., The N-terminal Ca2+-independent calmodulin-binding site on the inositol 1,4,5-trisphosphate receptor is responsible for calmodulin inhibition, even though this inhibition requires Ca2+,. Mol Pharmacol 66 (2004) 276‐284.
46. **Figure Legends**

**Figure 1: Cytosolic Ca2+ signals formed within the nanodomain within the membrane complex can be isolated using DM-BAPTA.** The membrane complex of human platelets is formed by the close apposition of the open canalicular system (OCS) and the main platelet Ca2+ store, the Dense Tubular System (DTS). The cytosolic space within the membrane complex forms an isolated region of cytosol in which a Ca2+ nanodomain may be formed in these cells. (Inset) DM-BAPTA buffers Ca2+ at high affinity to within around 30 nm of open ion channels, thus effectively buffering Ca2+ rises within the bulk cytosol, whilst leaving Ca2+ nanodomains formed at the mouth of open ion channels undisturbed. As the OCS and DTS are held within around 20 nm of each other, DM-BAPTA will not be able to prevent Ca2+ released from the DTS reaching the OCS membrane.Therefore by triggering IP3-mediated store depletion in the absence of extracellular Ca2+ we can isolate the Ca2+ nanodomain around the IP3 receptor. If the IP3R and NCX are closely-coupled at the membrane complex, then NCX-mediated Ca2+ removal should not be blocked by DM-BAPTA-loading, allowing for an indirect measurement of Ca2+ released into this nanodomain. Additionally, Ca2+-sensitive effectors such as calmodulin (CaM) may be able to bind Ca2+ and trigger Ca2+-dependent responses, in the absence of detectable Ca2+ rises in the bulk cytosol

**Figure 2:** **NCX-dependent Ca2+ removal from a cytosolic nanodomain can be detected in the absence of any detectable rise in bulk cytosolic Ca2+ concentration in human platelets** (A) Fura-2-loaded human platelets suspended in supplemented HBS were pre incubated with either 30 μM DM-BAPTA/AM, or an equivalent volume of its vehicle, DMSO, for 10 min at 37°C. Extracellular Ca2+ was then chelated with 1 mM EGTA and then 1 min later stimulated with 0.5 U.mL-1 thrombin. (B-E) NCX-mediated Ca2+ removal occurs in the absence of any detectable rise in bulk cytosolic Ca2+ concentration in DM-BAPTA-loaded human platelets. Washed human platelets suspended in supplemented HBS were pre-treated with 30 μM DM-BAPTA/AM (D,E), or an equivalent volume of its vehicle, DMSO (B,C), for 10 min at 37°C. Cells were also treated with either 50 μM KB-R7943 (C,E), or an equivalent volume of its vehicle, DMSO (B,D), for the last 5 min of this incubation. A final concentration of 2.5 μM Fluo-4 salt was then added to the extracellular medium immediately before the experiment. Extracellular Ca2+ was then chelated with 1 mM EGTA (not shown) and then 1 min later the cells were stimulated with 0.5 U.mL-1 thrombin. (F) NCX-dependent Na+ entry can be observed from the cytosolic Ca2+ nanodomain. SBFI-loaded human platelets suspended in supplemented HBS were preincubated with 30 μM DM-BAPTA/AM for 10 min at 37°C. Cells were also treated with either 50 μM KB-R7943, or an equivalent volume of its vehicle, DMSO for the last 5 min of this incubation. Extracellular Ca2+ was then chelated with 1 mM EGTA and 1 min later the cells were stimulated with 0.5 U.mL-1 thrombin*.*

**Figure 3: Ca2+ removal from the cytosolic nanodomain upon the normal subcellular localisation of the DTS.** (A) DM-BAPTA-loading prevents rises in the Ca2+ concentration of the bulk cytosol. Fura-2-loaded human platelets suspended in supplemented HBS were preincubated with either 30 μM DM-BAPTA/AM, or an equivalent volume of its vehicle, DMSO, for 10 min at 37°C. Extracellular Ca2+ was then chelated with 1 mM EGTA and then 1 min later stimulated with 0.5 U.mL-1 thrombin. (B,C) Nicergoline-induced disruption of the subcellular localisation of the DTS inhibits NCX-mediated Ca2+ removal from the cytosolic nanodomain in DM-BAPTA-loaded platelets. Washed human platelets were suspended in supplemented HBS were pre-treated with 30 μM DM-BAPTA/AM as well as either 100 μM nicergoline (B), or an equivalent volume of its vehicle, DMSO (C) for 10 min at 37°C. A final concentration of 2.5 μM Fluo-4 salt was then added immediately before the experiment. Extracellular Ca2+ was then chelated with 1mM EGTA (not shown) and then 1 min later the cells were stimulated with 0.5 U.mL-1 thrombin. (D) Nicergoline inhibits Ca2+ removal by the NCX at the cytosolic nanodomain. SBFI-loaded human platelets suspended in supplemented HBS were preincubated with 30 μM DM-BAPTA/AM and either 100 μM Nicergoline, or an equivalent volume of its vehicle, DMSO, for 10 min at 37°C. Extracellular Ca2+ was then chelated with 1 mM EGTA and 1 min later the cells were stimulated with 0.5 U.mL-1 thrombin.

**Figure 4: NCX-dependent Ca2+ removal from the cytosolic Ca2+ nanodomain generates pericellular Ca2+ hotspots.** (A)Pericellular Ca2+ hotspots are observed in the absence of any rise in bulk cytosolic Ca2+ concentration. Washed human platelets suspended in supplemented HBS were pre-treated with 30 μM DM-BAPTA/AM. Cells were also treated with either 50 μM KB-R7943 or an equivalent volume of its vehicle, DMSO, for the last 5 min of this incubation. Platelets were added to poly-lysine-coated chambered cover slides and allowed to adhere for 5 minutes. Extracellular Ca2+ was then chelated by addition of 1 mM EGTA and Fluo-4 salt was added at a final concentration of 2.5 μM. Cells were then stimulated with 0.5 U.mL-1 thrombin and observed for 5 minutes. (A) Representative images of the pericellular Ca2+ signals observed in DMSO-treated cells. Similar pericellular Ca2+ hotspots were seen in some cells treated with KB-R7943 but these were less frequent. (B) Thrombin-evoked pericellular signals are resistant to DM-BAPTA loading in untreated human platelets but are significantly less prevalent when pretreated with KB-R7943. Bar chart showing the percentage of cells showing pericellular Ca2+ hotspots during the recording period in DMSO- and KB-R7943-treated platelets co-loaded with DM-BAPTA and stimulated with thrombin.

**Figure 5: NCX3 is localised in a punctate pattern consistent with its localisation to the OCS membrane within the Membrane complex.** Washed fixed platelets were preincubated with a blocking buffer of PBS containing 1 mg.mL-1 BSA and a 1:100 dilution of a mouse secondary antibody for 30 min at room temperature. Cells were washed and then resuspended into PBS containing 1 mg.mL-1 BSA and either a 1:25 dilution of an anti-NCX3 primary antibody (NCX3) or no antibody (Primary-free) for 30 min at room temperature. Cells were then washed again and incubated in PBS containing 1 mg.mL-1 BSA and a 1:1000 dilution of a fluorophore-conjugated secondary antibody for 30 min at room temperature. Cells were washed and resuspended into PBS containing 1 mg.mL-1 BSA. The labelled cells were allowed to settle for 10 min on a poly-L-lysine –coated chambered slide. Fluorescent images were captured using a Fluoview FV 1200 laser scanning confocal microscope (Olympus, UK). NCX3 was principally found in 1-2 puncta localised just internal to the surface membrane in each platelet (yellow arrowheads). These are consistent with the known location of the membrane complex in human platelets [16,17]. These results suggesting that NCX3 is principally localised to the OCS membrane at the membrane complex.

**Figure 6: Inhibiting Ca2+ removal from the cytosolic nanodomain triggers platelet shape change, and enhances thrombin-evoked shape change.** Washed human platelets suspended in supplemented HBS were pre incubated with 30 μM DM-BAPTA/AM for 10 min at 37°C. Extracellular Ca2+ was then chelated with 1 mM EGTA. Cells were the treated with HBS containing either KB-R7943 or an equivalent volume of DMSO, to give a final concentration of either 25 μM (A) or 50 μM (B) KB-R7943. 2 min later cells were stimulated with 0.5 U.mL-1 thrombin. (C,D) Bar charts quantifying either the magnitude of the shape change response that occurs after KB-R7943 or DMSO addition prior to thrombin addition (C), or the degree of shape change maintained 5 minutes after thrombin stimulation (D). \*= p < 0.05 relative to the DMSO-treated control. † = p < 0.05 relative to both indicated conditions.

**Figure 7: Ca2+ rises within the cytosolic nanodomain are responsible for both the KB-R7943 and thrombin-evoked shape change in DM-BAPTA-loaded human platelets.** The effect of inhibitors of the Ca2+-dependent (ML-7) and the Ca2+-independent (Y-27632) shape change was investigated on KB-R7943 and thrombin-evoked shape change.Washed human platelets suspended in supplemented HBS were preincubated with 30 μM dimethyl-BAPTA/AM and either 25 μM ML-7 (A-D), 20 μM Y-27632 (E-H) or an equivalent volume of its vehicle, DMSO, for 10 min at 37°C. Extracellular Ca2+ was then chelated with 1 mM EGTA. Cells were the treated with HBS containing either KB-R7943 (B,F) or an equivalent volume of DMSO (A,G), to give a final concentration of 50 μM KB-R7943. 2 min later cells were stimulated with 0.5 U.mL-1 thrombin. Bar charts indicate the mean drug-induced shape change after KB-R7943 or DMSO addition prior to thrombin (C,G) or the degree of shape change maintained 5 minutes after thrombin stimulation (D,H). \* = p < 0.05 relative to DMSO/DMSO control. † = p < 0.05 relative to both conditions.

**Figure 8: Ca2+ release from the type 1 IP3 receptor is partly responsible for KB-R7943- and thrombin-induced shape change in DM-BAPTA-loaded platelets*.*** Washed human platelets suspended in supplemented HBS were preincubated with 30 μM dimethyl-BAPTA/AM for the 10 min at 37°C. For the last 5 min of this incubation cells were also treated with either 100 μM 2-APB or an equivalent volume of its vehicle, DMSO(A). Extracellular Ca2+ was then chelated with 1 mM EGTA. Cells were the treated with HBS containing either KB-R7943 or an equivalent volume of DMSO, to give a final concentration of 50 μM KB-R7943 (B). 2 min later cells were stimulated with 0.5 U.mL-1 thrombin. (C,D) Bar charts quantifying either the magnitude of the shape change response that occurs after KB-R7943 or DMSO addition prior to thrombin addition (C), or the time taken to reach the peak of the shape change response (D). \*= p < 0.05 relative to the DMSO-treated control. † = p < 0.05 relative to both indicated conditions.



**Figure 1**



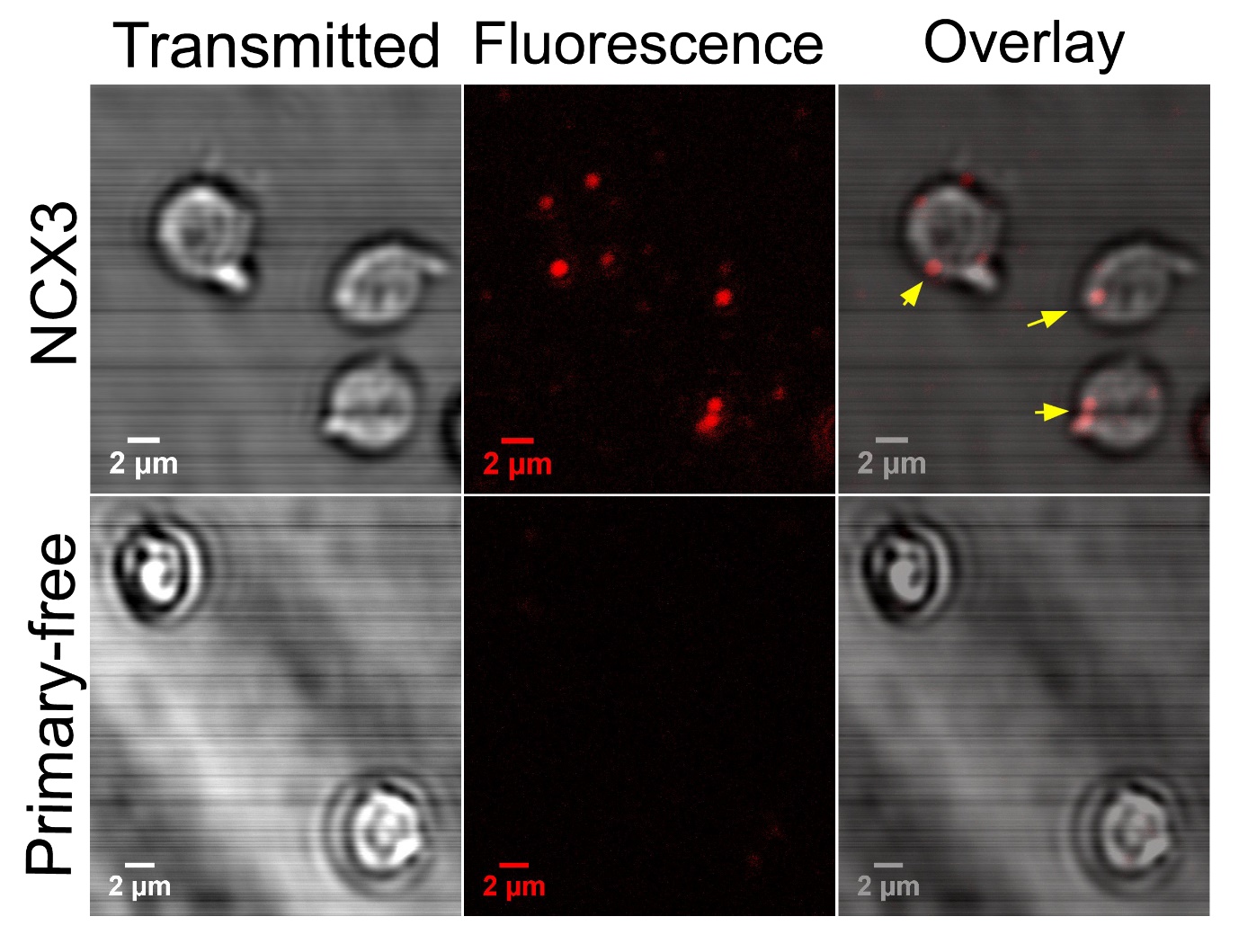
**Figure 2**

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**Figure 3**

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**Figure 4**

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**Figure 5**

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**Figure 6**

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**Figure 7**

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**Figure 8**

**Supplementary Materials**

**Human platelets use a cytosolic Ca2+ nanodomain to activate Ca2+-dependent shape change independently of platelet aggregation**

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1. **Methods**

**1.1 Normalisation of Fluo-4 and SBFI data**

Fluo-4 data was normalised by calculating F/F0 for all data points. F0 was defined as the mean Fluo-4 fluorescence recorded in the 5 seconds prior to thrombin addition. SBFI data was normalised by calculating the % of the maximum SBFI fluorescence achieved for all data points using the following formula:

% maximum SBFI fluorescence = 100 x (F – F0)/(Fmax – F0)

Where F0 was the mean SBFI fluorescence recorded for 5 seconds prior to thrombin addition, and Fmax was the maximum fluorescence measured after the addition of 50 µM gramicidin at the end of each experiment. Normalised Fluo-4 and SBFI data were quantified by integration of the change in the normalised signal from basal with respect to time for 3.5 min after thrombin addition.

1. **Results**

Normalisation of the Fluo-4 (Figure S1) and SBFI (Figure S2) data shown in the paper was found to have no effect on any of the findings from analysis of any of the unprocessed SBFI and Fluo-4 fluorescence traces shown in Figure 2 and 3 of the main paper. A side-by-side comparison of the statistical analysis of the unprocessed and normalised data analyses showed that this had no impact on any of the findings from the SBFI or Fluo-4 assays (Table S1).



**Table S1: Comparison of statistical results obtained from analysis of integration of our raw data and normalised data sets showed no impact on our findings.** \* indicates P < 0.05 relative to DMSO-treated control samples.



**Figure S1: Figure S2: Normalised versions of the Fluo-4 data shown in Figures 2B,C (A), Figure 2D,E (B) and Figure 3A,B (C) of the main paper.**



**Figure S2: Normalised versions of the SBFI data shown in Figure 2F (A) and 3C (B) of the main paper.**