Supporting Information: Nicergoline inhibits human platelet Ca^{2+} signalling through triggering a microtubule-dependent reorganisation of the platelet ultrastructure

<u>Additional Methods</u>

Materials

CytoPainter Phalloidin iFluor555 and Gö6976 were from Abcam (Cambridge, U.K.). Ro-0437626 was obtained from Tocris Bioscience (Bristol, U.K.). MeSAMP and MRS-2179 were from Sigma Aldrich (Gillingham, U.K.). Ketanserin was from Axxora (Nottingham, UK). All other reagents were of analytical grade.

F-actin Measurements

F-actin measurements were performed according to the method of Rosado *et* al., (2000). Briefly, unstimulated washed platelets were treated with either 100 µM nicergoline or its vehicle, DMSO, for 5 minutes at 37°C. Cells were then fixed by addition of 3% [w/v] formaldehyde and stored at 4°C until use. Platelets were collected by centrifugation and then permeabilised by incubation in Phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and 1 mg/mL bovine serum albumin for 10 minutes at room temperature. Cells were recollected by centrifugation and resuspended in PBS containing 1 µM CytoPainter Phalloidin iFluor555 and 1mg/mL BSA and incubated at room temperature for 30 minutes. Cells were recollected by centrifugation and finally resuspended in PBS containing 1 $mg.mL^{-1}$ BSA. 100 µL aliquots of each sample were plated into 96 well plates in either duplicate or triplicate. Fluorescence was then recorded using a BioTek Synergy 2 microplate reader using a 540 nm excitation wavelength and collected using a 590 nm emission filter. The mean of the replicates was then recorded for each sample.

Single platelet imaging

Fixed platelet samples for analysis of the actin structure of cells were prepared as above for the F-actin measurements. Platelet microtubule structure was monitored in cells loaded with 1 µM Fluo-5N/AM for 2 hours at 37°C in PRP (Sage *et al*., 2011). After dye loading cells were washed by centrifugation and resuspended in supplemented HBS. Cells were preincubated with 100 µM nicergoline (or its vehicle, DMSO) under magnetic stirring for 5 minutes at 37°C. Chambered slides (Ibidi µ-slide 8 well) were coated with a poly-L-lysine solution (Sigma Aldrich, UK) overnight at 4°C. Slides were washed with either supplemented HBS (Fluo-5N) or PBS (Phalloidin) and mounted on the microscope stage. Platelet suspensions at a density of 2×10^8 mL^{-1} were pipetted into the chambered coverslip and allowed to adhere to the substrate for 3 min. Excess platelet suspension was then removed and the slides washed twice with either supplemented EGTA to which 1 mM EGTA was added (Fluo-5N) or PBS (Phalloidin). Fluorescence was monitored using a Fluoview FV1200 laser-scanning confocal microscope (Olympus, U.K.) with a PLAPON 60× oil immersion objective. Images were recorded at a frequency of 0.5 Hz for 5 min excitation at 473 nm (Fluo-5N) or 543 nm (CytoPainter Phalloidin iFluo555) and emission at 490–520 nm or 590-620nm respectively.

Dense granule Secretion

For the Gö6976 experiments, luciferin-luciferase luminescence measurements were made using a using a BioTek Synergy 2 microplate reader on 100 µL aliquots of washed human platelet suspension dispensed into 96 well plates, to which 10% [v/v] luciferin-luciferase was added. Thrombin-evoked secretion was measured as the thrombin-evoked increase in

luminescence collected for 1 minute after thrombin addition when compared to the basal reading taken immediately prior to thrombin stimulation

Results

Nicergoline does not itself elicit a Ca^{2+} signal, but does trigger a small reduction in resting $[Ca^{2+}]_{cyt}$ in both the presence and absence of extracellular Ca^{2+}

Experiments were performed to examine the effect of nicergoline on resting $[Ca^{2+}]_{cyt}$ in cells treated in the presence and absence of extracellular Ca^{2+}. As shown in Supplementary Figure 1, addition of nicergoline itself elicited no increase in $[Ca^{2+}]_{cyt}$, but it did decelerate the rate of increase in the reported $[Ca^{2+}]_{cyt}$ in the presence of extracellular Ca^{2+} (Figure S1A; $\Delta[Ca^{2+}]_{cyt}$ = 15.3 ± 2.0 nM and 10.9 ± 2.2 nM for DMSO- and nicergoline-treated cells respectively; n = 5; $P < 0.05$) and accelerated the slow rate of decline seen in the absence of extracellular Ca^{2+} (Figure S2A,B; $\Delta[Ca^{2+}]_{cyt}$ = 2.3 ± 0.8 nM and 6.2 ± 0.8 nM for DMSO- and nicergoline-treated cells respectively; n = 5; $P < 0.05$) – consistent with a small but consistent effect of nicergoline on resting $[Ca^{2+}]_{cyt}$. In the experiments performed in the absence of extracellular Ca^{2+}, thrombin was added at the end of the 10 minute preincubation to act as a positive control of nicergoline activity. In these experiments nicergoline was found to reduce the thrombin-evoked rise in $[Ca^{2+}]_{cyt}$ to 76.7% ± 3.9% of control (n = 6; $P < 0.05$).

Nicergoline causes a slight thickening of the cortical F-actin layer without altering the polymerisation state of F-actin within the platelets

In addition to the tubulin cytoskeleton, platelets also contain an extensive actin cytoskeleton which plays a key role in triggering morphological changes upon activation and adhesion to the damaged vascular wall. Further experiments therefore examined the effect of nicergoline pretreatment on the F-actin content and distribution in human platelets. Cells were fixed, permeabilised and F-actin was labelled with fluorescently-labelled phalloidin. Measurements of F-actin content revealed that nicergoline induced no significant change in polymerised F-actin content in these cells (102.5% ± 7.7% of control; n = 5; $P > 0.05$), suggesting that nicergoline does not trigger active rearrangement of this cytoskeletal structure within the cells.

Platelets labelled with fluorescently-labelled phalloidin were examined using confocal microscopy to investigate the distribution of actin in nicergoline-treated cells. Fixed control platelets were found to principally exit in a discoid form, with cells seen to take a range of either thin discs or circular forms depending on which side of the platelet the cells had settled on the surface of the coverslide. In contrast, fixed nicergoline-treated platelets were found to exist as a near-homogenous population of rounded cells (Figure S2) suggesting that cells had changed from their discoid form into a spherical form, as previous observed by Le Menn *et al*., (1979). The observation that fixed nicergoline-treated platelets plated onto coverslips appear spherical, whilst live cells appear similar to the control cells, suggests that platelets are able to spread similarly to control cells through remodelling of their cortical F-actin layer.

Unlike its effect on the cortical microtubule bundle, nicergoline pretreatment was found to leave the cortical actin ring intact. However further examination of this structure found that the cortical F-actin layer was more pronounced in these cells compared to control cells (Figure 3B), with the cortical actin layer observed to be slightly thickened in nicergoline-treated platelets (308 nm ± 11 nm compared to 235 nm ± 6 nm in control cells; n = 5; $P < 0.05$). These results suggest that F-actin content is also redistributed in response to the nicergoline-induced disruption of the cortical microtubule bundle. This would be in line with previous work by Cerecedo *et al*., (2008), who found that disrupting actin or microtubule structure in platelets could simultaneously affect the organisation of the unaffected cytoskeletal component.

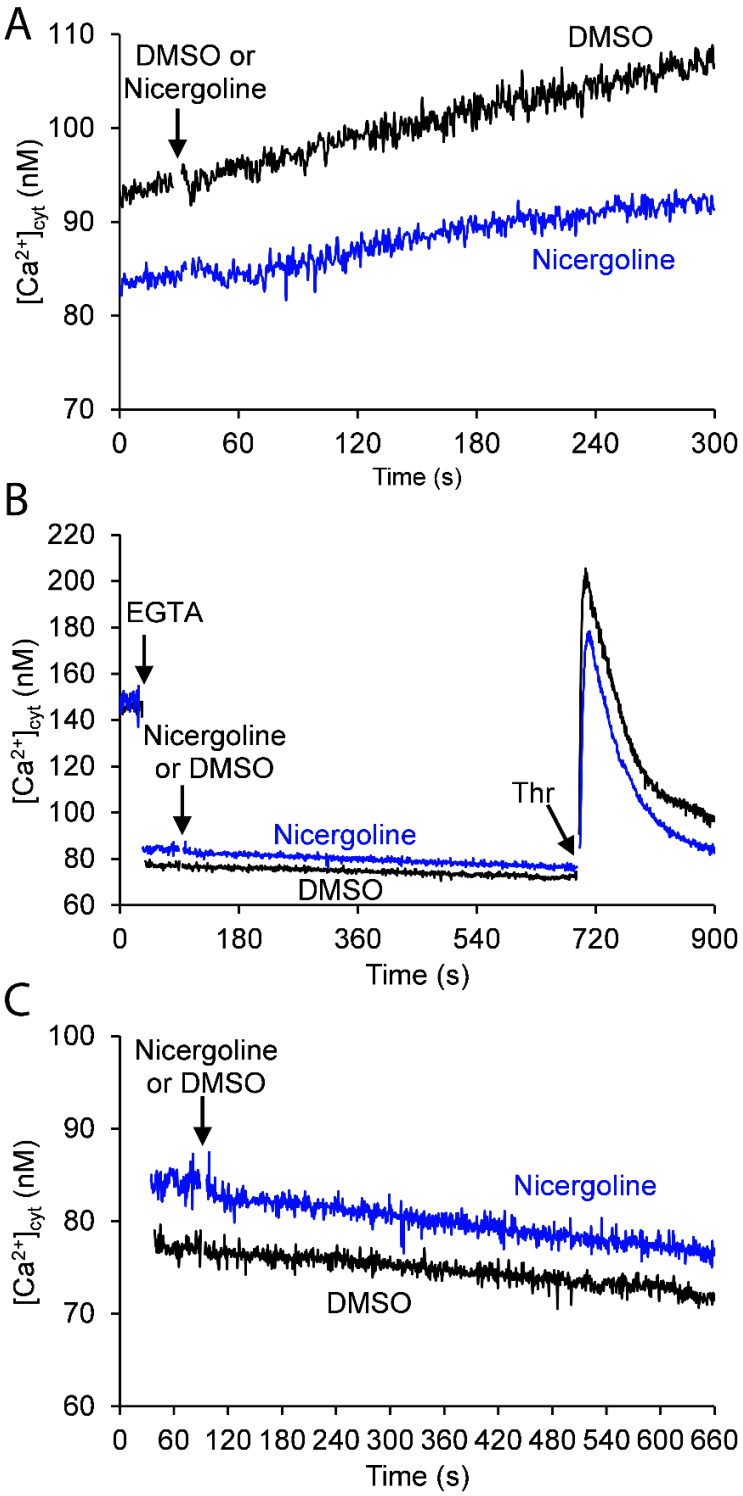

Figure S1: Nicergoline does not itself elicit a Ca^{2+} signal, but does trigger a small reduction in the baseline $[Ca^{2+}]_{cyt}$ in both the presence and absence of extracellular Ca^{2+}. Fura-2-loaded human platelets were suspended in supplemented HBS containing 200 µM $CaCl_2$ and prewarmed for 5 minutes at 37°C prior to testing. (A) Platelets were stimulated with 100 µM nicergoline or an equal volume of its vehicle, DMSO in the presence of continuous magnetic stirring. (B,C) 1 mM EGTA was added before the cells were treated with either 100 µM nicergoline or its vehicle for 10 min at 37°C in the presence of continuous magnetic stirring. Platelets were then stimulated with 0.5 $U.mL^{-1}$ thrombin. (C) is an expanded view of the signals observed after nicergoline treatment in (B). Results presented are representative of 5 and 6 experiments respectively.

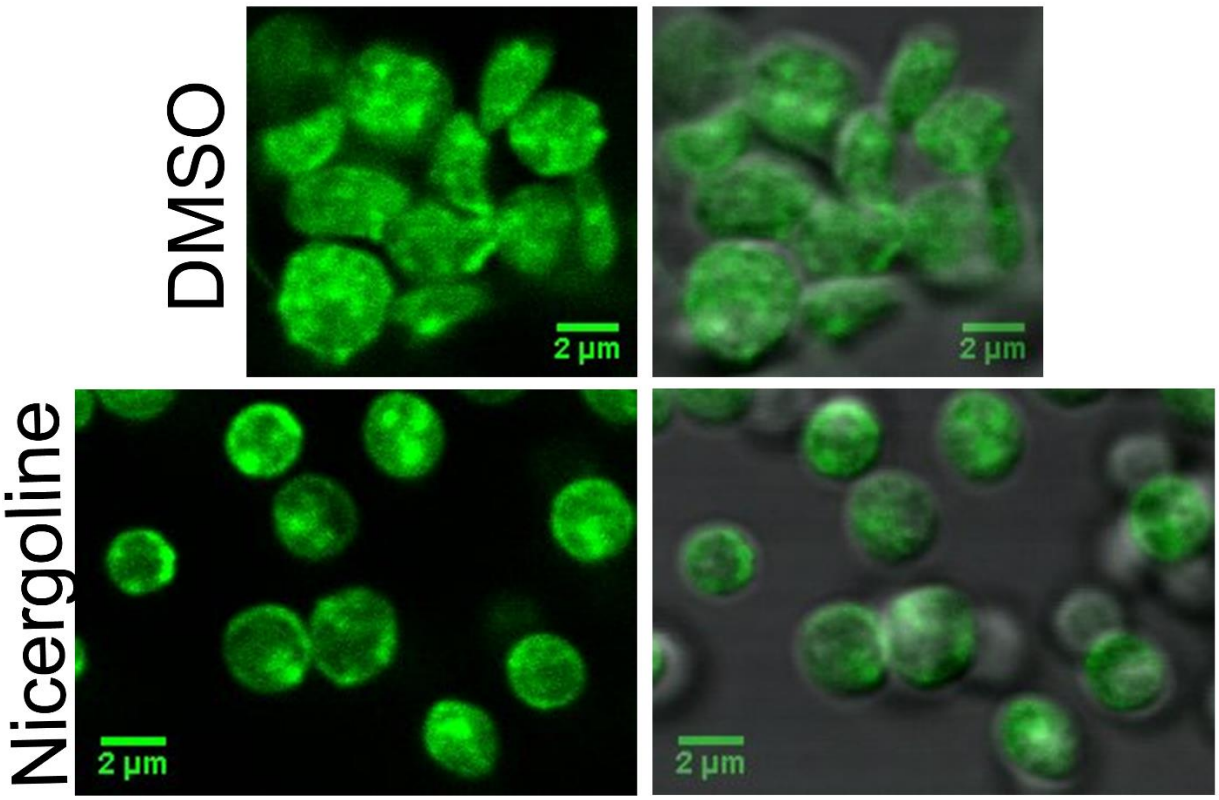

Figure S2: Nicergoline causes a slight thickening of the cortical F-actin layer without altering the polymerisation state of F-actin within the platelets. Platelets were treated with either DMSO (upper panels) or 100 µM nicergoline (lower panels) for 5 min at 37°C in the presence of continuous magnetic stirring. Cells were then fixed, permeabilised and incubated with Cytopainter Phalloidin iFluor555 to indicate the presence of F-actin. Platelets were washed and allowed to settle on poly-L-lysine coverslips for 5 minutes prior to imaging. Excess platelet suspension was removed and the slides were washed twice with Phosphate-buffered saline. Fluorescence was then monitored using an Olympus Fluoview FV1200 confocal microscope. The right-hand panels show the overlay of transmitted light image and fluorescence from the Cytopainter Phalloidin iFluor555; the lower panels show the fluorescence from TubulinTracker alone. The results presented are representative of 5 experiments.

Nicergoline triggers a reorganisation of the intracellular Ca^{2+} stores

The effect of nicergoline on intracellular Ca^{2+} store location, was monitored in resting Fluo-5N-loaded platelets treated with either nicergoline or DMSO. Consistent with our previous findings (Sage *et al*., 2011), control platelets were observed to have an inhomogeneous, punctate distribution of intracellular Ca^{2+} stores (Figure S3A,B). In contrast, nicergoline-treated platelets had a more homogenous Ca^{2+} store distribution with no obvious punctae of raised fluorescence observed (Figure S3C,D). Homogeneity of the Fluo-5N fluorescence under both conditions was quantitatively assessed by comparing the variance in Fluo-5N pixel fluorescence from treated and untreated cells, with the mean standard deviation being 8.4 ± 0.2 arbitrary units for nicergoline-treated platelets compared to 12.8 ± 0.6 arbitrary units in DMSO-treated platelets (both $n = 8$; $P < 0.05$). The loss of punctae in nicergoline-treated cells lead to a reduction in the maximum pixel fluorescence of the cell when compared to that observed in DMSO-treated cells (63.9% ± 1.6% of control; $n = 8$; $P < 0.05$). In contrast mean Fluo-5N fluorescence of the cells were found to be unaffected (96.3% ± 13.9% of control; $n = 8$; $P > 0.05$). A linescan analysis of 3 of the cells shown was also performed (Figure S3E) – these showed that whilst there was a comparable basic level in the nicergoline- and DMSO-treated cells, there were one or two significant peaks in Fluo-5N fluorescence observed in every DMSO-treated cell that were not seen in nicergoline-treated cells. These data therefore suggest that whilst the amount of Ca^{2+} stored inside these cells is not altered by nicergoline treatment, the distribution of the stored Ca^{2+} is significantly altered, presumably due to microtubule-associated reorganisation of the DTS.

Nicergoline elicits no additional inhibitory effect when pericellular Ca^{2+} accumulation is prevented by pretreatment with an NCX inhibitor.

In our previous study we have demonstrated that pericellular Ca^{2+} accumulation is dependent upon the forward mode exchange activity of the NCX (Sage *et al*., 2013). If nicergoline works via interrupting pericellular Ca^{2+} recycling in human platelets, blocking the NCX with its inhibitor KB-R7943, should prevent further nicergoline-induced inhibition of the thrombin-evoked Ca^{2+} signal. As shown in Figure S4, Ca^{2+} release as previously observed (80.4% ± 1.9% of control and 32.2% ± 3.5% of control for nicergoline or KB-R7943 alone; n = 6; $P < 0.05$). Pretreatment with both inhibitors together did not reduce the thrombin-evoked Ca^{2+} signal further when compared to the effect KB-R7943 alone (43.6% ± 4.3% of control; n = 6; $P < 0.05$ when compared to both control cells and cells treated with KB-R7943 alone). Our data showed that nicergoline treatment alongside KB-R7943 actually significantly potentiated the thrombin-evoked Ca^{2+} signal seen in NCX-inhibited cells. These data suggests that nicergoline inhibits thrombin-evoked rises in $[Ca^{2+}]_{cyt}$ by reducing the efficacy of the pericellular recycling system.

The nicergoline-induced potentiation of the Ca^{2+} signals in KB-R7943-treated cells was an unexpected finding and suggests that redistribution of the intracellular stores may delocalise Ca^{2+} release from the dense tubular system from another Ca^{2+} removal or sequestration mechanism preventing effective Ca^{2+} transport out of the cytosol. We have previously suggested that the plasma membrane Ca^{2+}-ATPase (PMCA) may contribute to pericellular recycling as this transporter is localised within the OCS in close proximity to the DTS in platelets (Cutler *et al*., 1980), and treatment with PMCA inhibitors reduced agonist-evoked Ca^{2+} signals and dense granule secretion (Jones *et al*., 2010). In nicergoline-treated cells, dissociation of the DTS from the OCS may therefore also reduce the efficacy of the PMCA in removing Ca^{2+} following thrombin-evoked Ca^{2+} rises. Further work will be needed to examine this hypothesis further.

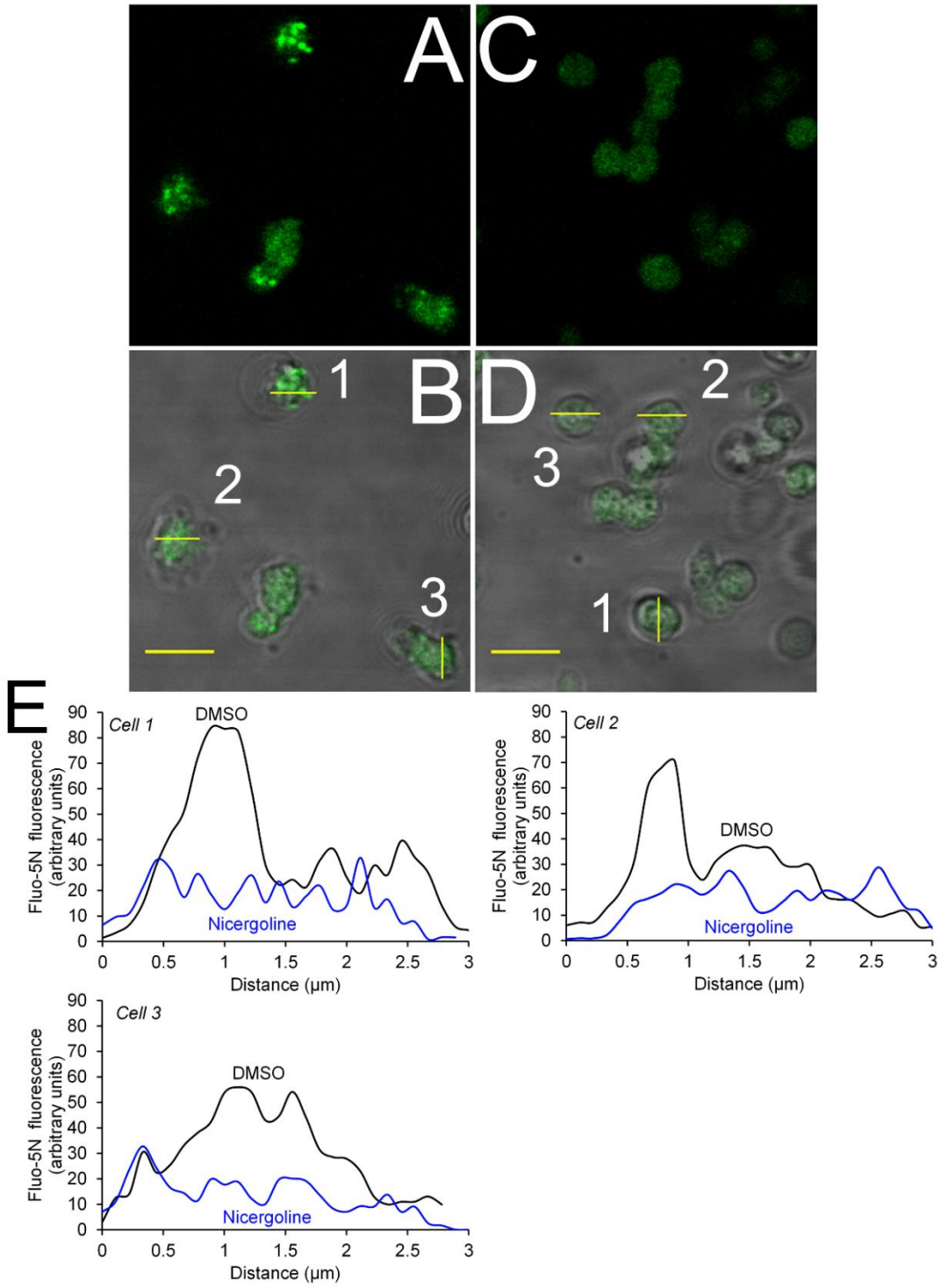

Figure S3: Nicergoline triggers reorganisation of the subcellular location of the intracellular Ca^{2+} stores. Fluo-5N-loaded platelets were suspended in supplemented HBS. Cells were pretreated with either DMSO (A,B) or 100 µM nicergoline (C,D) for 5 min at 37°C in the presence of continuous magnetic stirring. The platelets were then added to a poly-L-lysine-coated chambered slide, and allowed to settle for 3 minutes. Excess platelet suspension was removed and the slides were washed twice with Ca^{2+}-free HBS. Fluorescence was then monitored using an Olympus Fluoview FV1200 confocal. Images for Fluo-5N alone (A,B) or overlaid with the transmitted light image (C,D) are shown. Scale bar in bottom left corner indicates 5 µm. (E) A Linescan analysis of the 3 numbered cells indicated in (B,D). The yellow lines indicated where the line scan analysis was performed. Results of representative of 8 experiments.

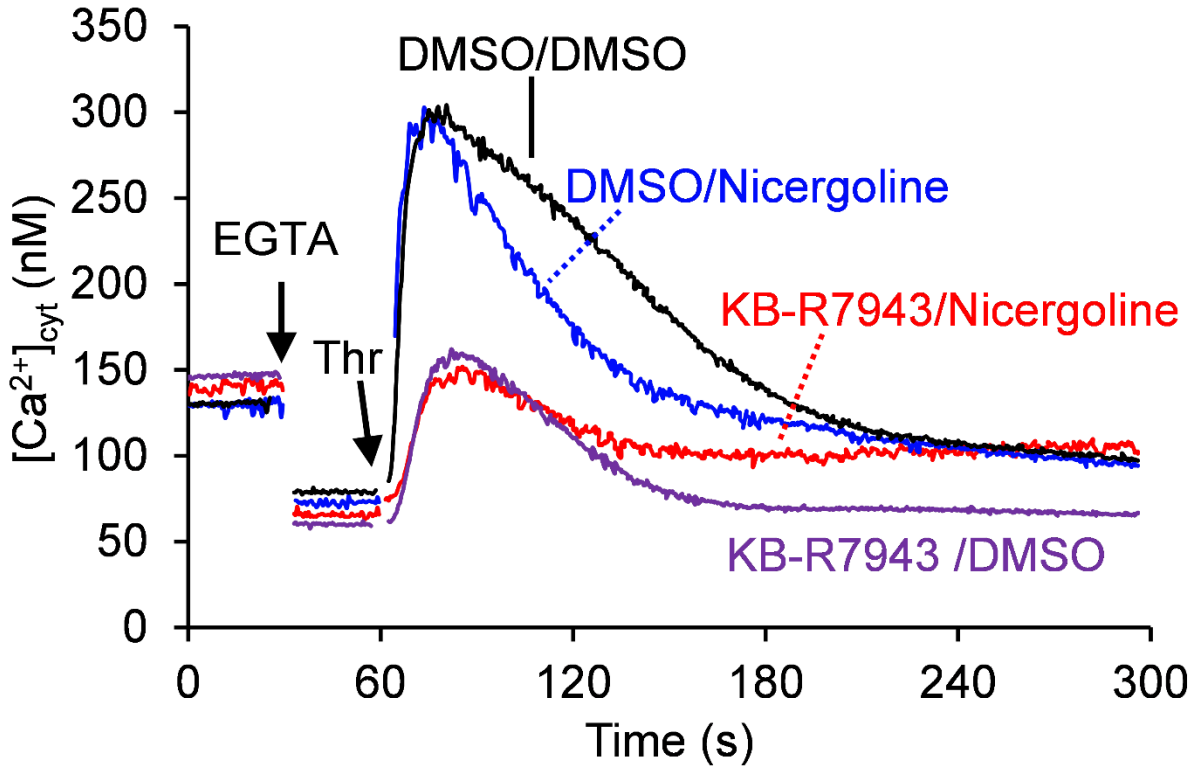

Figure S4: Nicergoline elicits no additional inhibitory effect when pericellular Ca^{2+} accumulation is prevented by pretreatment with an NCX inhibitor. Fura-2-loaded human platelets were suspended in supplemented HBS. Platelets were pre-treated with either 100 µM nicergoline or an equal volume of its vehicle (DMSO) for 5 min at 37°C in the presence of continuous magnetic stirring. Cells were then treated with either 50 µM KB-R7943 for 5 additional minutes at 37°C. 1 mM EGTA was then added before cells were stimulated with 0.5 $U.mL^{-1}$ thrombin. The results presented are representative of 6 experiments

The inhibitory effect of nicergoline on thrombin-evoked Ca^{2+} signalling is not caused by a reduction in autocrine signalling.

Previously, our group and others have found that blocking autocrine-mediated signalling can reduce thrombin-evoked Ca^{2+} release (Lages and Weiss, 1999; Sage *et al*., 2013). The finding that nicergoline can significantly reduce thrombin-evoked dense granule secretion, therefore raises the question as to whether reduced dense granule secretion is the cause, and not the effect, of the reduced thrombin-evoked Ca^{2+} signal. If thrombin-evoked Ca^{2+} signalling was dependent upon the IP_3-mediated release of Ca^{2+} from intracellular stores triggered by ADP or serotonin, then blocking autocrine signalling in these cells should prevent thrombin-evoked Ca^{2+} release. As can be seen in Figure S5A, combined inhibition of P_{2X1}, 5-HT_{2A}, P_{2Y1} and P_{2Y12} receptors was found to potentiate and not inhibit, the net release of Ca^{2+} from intracellular stores (128.8% ± 3.4% of control; n = 6; $P < 0.05$). These results suggest that autocrine stimulation of platelets is not responsible for facilitating thrombin-evoked Ca^{2+} release from the intracellular stores. Instead the greater depletion is indicative of a failure to refill the Ca^{2+} stores as effectively – hence leading to the increased depletion observed here. In addition, as nicergoline inhibits the primary Ca^{2+} release, whilst autocrine signalling blockers do not, these results suggest that nicergoline works to principally inhibit thrombin-evoked Ca^{2+} signalling, which as a consequence prevents dense granule secretion.

These results do beg the question on what role autocrine signalling does play in mediating thrombin-evoked Ca^{2+} signalling. Previous work on human subjects with delta storage pool disorder demonstrated that autocrine stimulation of platelets was principally required to maintain the opening of divalent cation channels (Lages & Weiss, 1999). In addition, we demonstrated that preventing pericellular recycling of Ca^{2+} back into the cytosol by blocking non-selective cation channels could reduce thrombin-evoked rises in $[Ca^{2+}]_{cyt}$, as well as prevent refilling of intracellular Ca^{2+} stores leading to them becoming more depleted (Sage *et al*., 2013). Therefore Mn^{2+} quench experiments were performed to examine whether blocking autocrine stimulation of platelets prevented opening of non-selective cation channels in thrombin-stimulated platelets. As shown in Figure S5B, combined inhibition of all autocrine signalling reduced thrombin-evoked Mn^{2+} quench to 78.4± 4.3% of control (n = 6; $P < 0.05$). These results confirms that autocrine signalling does not mediate its effect on thrombin-evoked signalling through assisting in the depletion of the intracellular Ca^{2+} stores, but instead works to open Ca^{2+}-permeable ion channels to allow Ca^{2+} recycling to contribute to the cytosolic Ca^{2+} signal, which in turn facilitates intracellular Ca^{2+} store refilling. These results therefore demonstrate that nicergoline's inhibitory effect on thrombin-evoked Ca^{2+} signalling is not principally due to loss of autocrine signalling from the platelet dense granules.

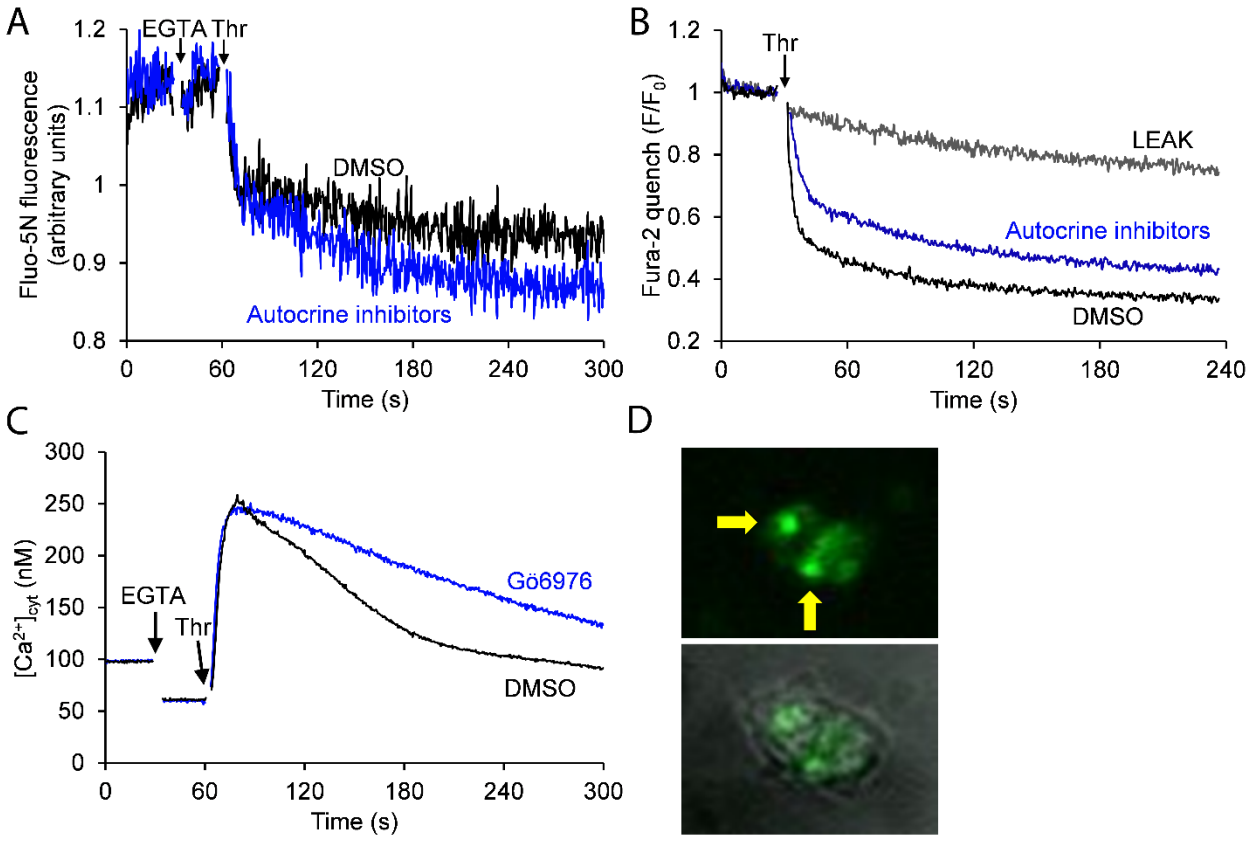

Figure S5: Nicergoline-induced inhibition of thrombin-evoked Ca^{2+} signalling is a cause, and not a consequence, of the nicergoline-related inhibition of dense granule secretion. (A)Fluo-5N-loaded human platelets were suspended in supplemented HBS. Platelets were pre-treated with either the autocrine inhibitors (25 µM Ro-0437626, 50 µM Ketanserin, 75 µM MRS-2179 and 100 µM MeSAMP), or an equal volume of their vehicles, DMSO, for 5 min at 37°C. 1 mM EGTA was added before the cells were stimulated with 0.5 $U.mL^{-1}$ thrombin. The results presented are representative of 6 experiments. (B) Fura-2-loaded platelets suspended in Ca^{2+}-free HBS supplemented with 0.1 $U.mL^{-1}$ apyrase were preincubated with either the autocrine inhibitors (25 µM Ro-0437626, 50 µM Ketanserin, 75 µM MRS-2179 and 100 µM MeSAMP), or an equal volume of their vehicles, DMSO, for 5 min at 37°C. Extracellular Ca^{2+} was chelated by addition of 500 µM EGTA, followed 30 seconds later by 1 mM $MnCl_2$. Platelets were stimulated 30s later with 0.5 U mL^{-1} thrombin. (C) Fura-2-loaded human platelets were suspended in supplemented HBS. Platelets were pre-treated with either 3 µM Gö6976 for 10 min at 37°C or an equal volume of its vehicle (DMSO) for 10 min at 37°C. 1 mM EGTA was then added before cells were stimulated with 0.5 $U.mL^{-1}$ thrombin. The results presented are representative of 6 experiments. (D) Cells were pretreated with 3 µM Gö6976 for 10 min at 37°C. The platelets were then added to a poly-L-lysine-coated chambered slide, and allowed to settle for 3 minutes. Excess platelet suspension was removed and the slides were washed twice with Ca^{2+}-free HBS containing 1 mM EGTA and 2.5 Fluo-4 K^+ salt, and platelets were then stimulated by addition of 0.5 $U.mL^{-1}$ thrombin. Fluorescence was then monitored using an Olympus Fluoview FV1200 confocal microscope at 0.5Hz for 5 minutes. The image shows 2 Gö6976-treated cells both showing the simultaneous expression of pericellular Ca^{2+} hotspots (yellow arrows) (A-D) The results presented are representative of 6 experiments.

Ca^{2+} accumulation in the pericellular Ca^{2+} hotspot occurs in the absence of dense granule secretion.

As platelet dense granules are known to contain a high concentration of Ca^{2+} (Ruiz *et al.*, 2004), nicergoline inhibition of the pericellular Ca^{2+} hotspot, and thus thrombin-evoked Ca^{2+} release, might be due to the cells inability to exocytose Ca^{2+} into the pericellular space. Previous work has demonstrated that the conventional PKC isoform-selective inhibitor Gö6976 can abolish thrombin-evoked dense granule secretion (Strehl *et al*., 2007). Experiments were therefore performed to examine the effect of the conventional PKC inhibitor Gö6976 on thrombin-evoked dense granule secretion and cytosolic and pericellular Ca^{2+} signalling elicited in the absence of extracellular Ca^{2+}. Prior to the performance of Ca^{2+} signalling studies, the effect of Gö6976 on thrombin-evoked dense granule secretion was tested using a luciferin-luciferase assay. Pretreatment of washed platelet suspensions with 3 μM Gö6976 for 10 minutes at 37°C was found to essentially abolish dense granule secretion (6.8% ± 3.3% of control; n = 5; $P < 0.05$). Thrombin-evoked Ca^{2+} release was also found to be significantly potentiated under these conditions in fura-2-loaded platelet samples from the same donor (178.6 ± 19.3% of control; n = 10; $P < 0.05$; Figure S5C), suggesting that Ca^{2+} removal and sequestration systems were both inhibited in line with to other recent findings (Lever *et al*., *manuscript in submission*). If Ca^{2+} exocytosis from the dense granules into the pericellular space was a prerequisite for Ca^{2+} store mobilisation into the cytosol via pericellular recycling, then Gö6976 would be expected to significant inhibit, and not enhance, the Ca^{2+} signal observed. These data therefore suggest that intracellular Ca^{2+} release from the intracellular stores occurs only directly via Ca2+ exit from the stores through open IP_3 and NAADP receptors (Sage *et al*., 2011), and not indirectly via the extracellular space.

Despite the effect of Gö6976-treated on the thrombin-evoked cytosolic Ca^{2+} signal, cells could be seen to form pericellular hotspots in 81.6 ± 5.5% of Gö6976-treated cells which was not significantly different to the 92.3% ± 2.3% observed in DMSO-treated cells (Figure S5D; n = 6; $P > 0.05$). Although these hotspots were seen on half of the occasions to have a notable reduction in fluorescent intensity, this effect was inconsistent and only seen in 3/6 experiments. However despite this, the continued appearance of pericellular hotspots in all samples of Gö6976-treated cells suggest that dense granule secretion is not principally responsible for the formation of the pericellular Ca^{2+} hotspot.

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