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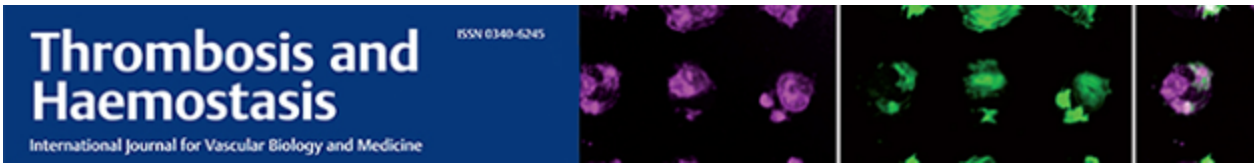

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Adenosine and forskolin inhibit platelet aggregation by collagen but not the proximal signalling events

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Abstract

Background: The G protein-coupled receptor, adenosine A_{2A}, signals through the stimulatory G protein, G_s in platelets leading to activation of adenylyl cyclase and elevation of cAMP and inhibition of platelet activation.

Objective: To investigate the effect of A_{2A} receptor activation on signalling by the collagen receptor glycoprotein (GP) VI in platelets.

Methods: Washed human platelets were stimulated by collagen or the GPVI-specific agonist collagen-related-peptide (CRP) in the presence of the adenosine receptor agonist, 5'-N-ethylcarboxamidoadenosine (NECA) or the adenylyl cyclase activator, forskolin and analysed for aggregation, ATP secretion, protein phosphorylation, spreading, Ca^{2+} mobilisation, GPVI receptor clustering, cAMP, TxB_2 and P-selectin exposure.

Results: NECA, a bioactive adenosine analogue, partially inhibits aggregation and secretion to collagen or CRP in the absence or presence of the $P2Y_{12}$ receptor antagonist, cangrelor and the cyclooxygenase inhibitor, indomethacin. The inhibitory effect in the presence of the three inhibitors is largely overcome at higher concentrations of collagen but not CRP. Neither NECA nor forskolin altered clustering of GPVI, elevation of Ca^{2+} or spreading of platelets on a collagen surface. Further, neither NECA nor forskolin, altered collagen induced tyrosine phosphorylation of Syk, LAT nor PLCγ2. However, NECA and forskolin inhibited platelet activation by the thromboxane A_2 (TxA_2) mimetic, U46619, but not the combination of ADP and collagen.

Conclusion: NECA and forskolin have no effect on the proximal signalling events by collagen. They inhibit platelet activation in a response specific manner in part through inhibition of the feedback action of thromboxane A_2.

Key words: adenosine, cAMP, collagen, glycoprotein VI (GPVI) receptor, platelets

Summary Table

What is known about this topic?
• Adenosine stimulates cAMP formation leading to inhibition of platelet activation by collagen. • There are contrasting reports on whether the proximal signalling events induced by collagen are altered in the presence of cAMP elevation.
What this paper adds?
• Adenosine has no effect on the proximal events in the GPVI signalling cascade up to intracellular Ca^{2+} but inhibits the feedback action of downstream signals including thromboxane A_2.

Introduction

Platelets are small anucleate cytoplasmic discs derived from megakaryocytes in the bone marrow that circulate in the blood. They play a vital role in the control of haemostasis and thrombus formation as well as in supporting inflammation, immunity, angiogenesis and vascular integrity[1]. Platelets are exposed to prostacyclin and nitric oxide at the endothelial surface which leads to elevation of cAMP and cGMP, respectively[2]. This is the most potent endogenous mechanism of platelet inhibition[3]. cAMP and cGMP activate the cyclic nucleotide-dependent protein kinases, PKA and PKG respectively, leading to phosphorylation of a wide variety of substrate proteins[4]. The consequences of phosphorylation include inhibition of Ca^{2+} release from intracellular stores[5], modulation of cytoskeletal actin dynamics via vasodilator-stimulated phosphoprotein (VASP), LIM and SH3 domain protein (LASP), heat shock protein (HSP) 27, filamin-A and caldesmon[6-8], and inhibition of heterotrimeric and small G proteins. This leads to inhibition of integrin activation, secretion and adhesion[9].

Collagen binds to the immunoglobulin receptor glycoprotein (GP) VI which is its major signalling receptor in platelets, and to integrin $\alpha_2\beta_1$ which supports platelet adhesion[10]. GPVI forms a complex in the membrane with the FcRγ-chain which contains an immunoreceptor tyrosine-based activation motif (ITAM) defined by the presence of two YxxL groups (single amino acid code) separated by 12 amino acids[11]. Collagen binds selectively to dimeric GPVI[12] and induces phosphorylation of the conserved tyrosine residues in the ITAM through the action of Src family kinases. This leads to binding of the tyrosine kinase Syk through its tandem SH2 domains and initiation of a downstream signalling pathway that culminates in activation of PLCγ2[10]. Collagen activates platelets at sites of vascular lesions and so it is important to understand how collagen is able to do this in the presence of elevated levels of cyclic nucleotides.

Adenosine is an endogenous nucleotide that inhibits platelet activation by ADP and collagen through A_{2A} and A_{2B} receptors. Both receptors activate the heterotrimeric G protein subunit, G_s, leading to elevation of cAMP[13-15]. Adenosine has been reported to inhibit platelet aggregation, ATP secretion, P-selectin cell surface expression and adhesion to a collagen surface[14] and to inhibit thrombus formation *in vivo*[14]. The inhibitory action of adenosine may contribute to the clinical efficacy of the $P2Y_{12}$ receptor antagonist, ticagrelor, which inhibits uptake of adenosine into platelets[16].

There are also reports that cAMP does not inhibit platelet activation by collagen. For example, prostacyclin which increases cAMP levels, has been reported to inhibit thrombin but not collagen induced tyrosine phosphorylation and signalling[17-19]. In addition collagen induced shape change, which is mediated by Ca^{2+} mobilisation, is insensitive to cAMP [20]. On the other hand, Loyau et al[21] reported that elevation of cAMP inhibits binding of a monoclonal antibody (mAb), 9E18, that binds selectively to dimeric GPVI, and Takayama et al[22] reported that cAMP

promotes endocytosis of GPVI. Both actions should result in inhibition of platelet activation by collagen.

In view of these contrasting reports, the aim of the present study was to investigate the effect of adenosine and forskolin which is a more powerful stimulus of adenylyl cyclase, on platelet activation by collagen. We report that adenosine and forskolin inhibit platelet aggregation by low concentrations but not high concentrations of collagen downstream of the proximal signalling events including elevation of intracellular Ca^{2+}.

Methods

Materials

Horm collagen was obtained from Takeda (High Wycombe, UK). NECA (5'-N-ethylcarboxamidoadenosine) was purchased from Tocris Bioscience (Bristol, UK). Cangrelor was purchased from Medicines Company. U46619 was obtained from Cayman Chemical. Chronolume and ATP were from Chrono-log Corporation (Manchester, UK). Horseradish peroxidase (HRP)-conjugated secondary antibodies and Enhanced Chemiluminescence (ECL) substrate were obtained from Amersham Biosciences (GE Healthcare, Bucks, UK). Oregon green 488 BAPTA-1-AM was purchased from Invitrogen (Invitrogen, ThermoFisher Scientific, Paisley, UK). Other reagents were obtained from Sigma.

Antibodies: PLCγ2 p1217, PLCγ2 p759, Syk p525/526, Syk p323, Syk p352, LAT p132 and LAT p171 were from Cell Signalling Technology (Danvers, Massachusetts, United States). LAT p200 mAb was obtained from Abcam (Cambridge, UK). Mouse anti-human anti-phosphotyrosine (clone 4G10) mAb was from Millipore UK Ltd (Watford, UK). Alexa Fluor 488 phalloidin and Alexa Fluor 647 were purchased from Invitrogen (Invitrogen, ThermoFisher Scientific, Paisley, UK). 1G5-Fab recognises monomeric and dimeric GPVI was raised as described[23].

Preparation of human washed platelets

Blood was collected from healthy and consenting volunteers by venipuncture in accordance with the Declaration of Helsinki (local ethical review no: ERN_11-0175). Trisodium citrate [1 part 3.8% (w/v) stock: 9 parts blood] was used as the anticoagulant. Washed platelets were prepared by centrifugation in the presence of prostacyclin (2.8 μM) followed by resuspension in Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$ 20 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose, pH 7.3) as previously described[24]. Platelet count was measured using a Coulter counter and the suspension was diluted to the required concentration in Tyrodes-HEPES buffer. Platelets were then left for 30 min. For aggregation and ATP secretion measurements platelets were used at $2x10^8$/ml; for western blotting at $5x10^8$/ml and for spreading at $2x10^7$/ml.

Aggregometry and luminescence measurement of ATP secretion

Aggregation was monitored using a Chrono-Log optical aggregometer (Labmedics, Manchester, UK) at 37°C with constant stirring at 1200 rpm. Secretion of ATP was measured using luciferin-luciferase Chrono-lume reagent (Chrono-Log). NECA (100 μM), indomethacin (10 μM), cangrelor (10 μM), forskolin (10 μM), ADP (10 μM) or vehicle were added 60-120 sec before collagen, CRP or U46619. The platelets were left for 5 min before addition of ATP (2 nM) for calibration of ATP secretion.

Platelet lysis and protein phosphorylation

Whole cell lysates were prepared from 400 μl sample of stirred platelet suspensions. Eptifibatide (9 μM) was added to prevent aggregation. The reaction was terminated by the addition of 5x SDS sample buffer (reducing conditions) to the platelets. The samples were heat denatured at 100°C for 5 min and spun at 15000 *g* for 10 min. The whole cell lysate was electrophoresed on 4-12% BisTris Plus acrylamide Bolt gels (Invitrogen, Paisley, UK) and

transferred onto PVDF membranes using a Trans-blot Transfer Imaging system (Bio-Rad, Hertfordshire, UK). Each gel included at least one lane of 10 µl of Color Prestained Protein Standard (11–245 kDa; NEB, Ipswich, Massachusetts, United States). Membranes were blocked in 4% (w/v) BSA dissolved in TBST and 0.1% (w/v) sodium azide for a minimum of 1 h at room temperature and incubated overnight at 4°C with anti-phosphotyrosine (clone 4G10; 1:1,000), PLCγ2 (Tyr 1217; 1:250, Tyr 759; 1:1,000), Syk (Tyr 525/526; 1:500, Tyr 352; 1:1,000, Tyr 323; 1:1,000) and LAT (Tyr 200; 1:500, Tyr 171; 1:1,000, Tyr 132; 1:500) primary antibodies. Membranes were imaged using the Licor Odyssey system. Quantification of band intensities was performed using Image Studio Lite v5.2 where background correction was applied.

Human platelet spreading

Glass coverslips were coated with collagen (10 µg/ml) in manufacturer-supplied diluent overnight at 4°C. The coverslips were blocked with 5 mg/ml BSA in PBS (heat denatured, filter-sterilised) for 1 h at room temperature, then washed with PBS. Washed platelets were incubated on collagen for 30 min at 37°C and NECA and forskolin were added and left for an additional 30 min. Pre-incubation experiments were conducted where NECA and forskolin were incubated with platelets for 30 min and followed by 30 min spreading. Following spreading the coverslips were washed in PBS and adherent platelets were fixed in 4% (w/v) formalin for 10 min at room temperature, permeabilized with 0.1% (v/v) Triton X-100 (in PBS) and stained with phalloidin-Alexa Fluor 488. The coverslips were mounted and imaged with a Zeiss Observer 7 Epifluorescent microscope using a 63x 1.4NA oil immersion lens. Images were acquired using Zen Pro v2.3 and processed using FIJI v1.51. Using the open-source KNIME software[25] platelet segmentation was performed and platelet count and surface area were analysed. An ilastik[26] pixel classifier was used to produce a binary segmentation. To separate touching platelets the centre of individual platelets were manually selected using

KNIME. These centre coordinates were then used as seeds for a watershed transform to produce the final segmentation result. Objects smaller than 1 μm^2 were discarded and platelet statistics including platelet area were calculated.

Single cell Ca^{2+} measurements

For Ca^{2+} measurements, washed platelets were incubated for 45 min at 37°C with Oregon green 488 BAPTA-1-AM (1 μM) in Tyrodes-HEPES buffer and centrifuged at 1000 *g* for 10 min with prostacyclin (2.8 μM) and resuspended in Tyrodes-HEPES buffer. Glass bottomed MatTek dishes (No. 1.5 (0.17 mm) coverslips) (MatTek, Ashland, MA, USA) were prepared as previously described[27]. Washed platelets were prepared and left to rest for 30 min. The platelets were diluted to $2x10^7$/ml and prior to exposure to an immobilized collagen surface were incubated with forskolin or indomethacin and cangrelor or all three inhibitors for 2 min. Real-time platelet Ca^{2+} flux was monitored using a Zeiss Observer 7 Epifluorescent microscope using a 63x 1.4NA oil immersion lens, Colibri 7 LED light source, Zeiss Filter set 38 for GFP/FITC, and Hammamatsu ORCA Flash 4 LT sCMOS camera where images were taken every 2 sec for 100 cycles. Images were acquired using Zen Pro v2.3 and processed using FIJI v1.51. The number of peaks and peak fluorescence intensity were analyzed in 75 platelets for each condition. Ca^{2+} spikes were identified where an increase in fluorescence intensity greater than 100 (a.u.) above baseline was observed. Where prolonged Ca^{2+} signals were detected, peaks were subdivided into multiple peaks when a clear change in direction of the trace was observed.

Total internal reflection microscopy (TIRFM)/direct STocastic Optical Reconstruction Microscopy (dSTORM)

Glass bottomed MatTek dishes (No. 1.5 (0.17 mm) coverslips) were coated with collagen (10 μg/ml) as previously described[27]. 1G5 Fab (pan-GPVI)-labelled (2 μg/ml) washed platelets

(2x10^7/ml) were allowed to adhere and spread for 30 min to the collagen-coated MatTek dishes. NECA (100 μM) and forskolin (10 μM) were added and platelets were spread for an additional 30 min. Adherent platelets were fixed in 4% (w/v) formalin for 10 min at room temperature, permeabilized with 0.1% (v/v) Triton X-100 (in PBS), blocked for 30 min in 2% (v/v) goat serum and 1% BSA (w/v) and then stained with phalloidin-Alexa Fluor 488 and Alexa Fluor 647-conjugated IgG secondary antibodies (diluted 1:300 in block buffer). Adhesion of 1G5 Fab-labelled washed platelets (2x10^7/ml) to immobilised collagenous substrate was imaged in TIRF using a fully motorised Nikon TIRF combine mounted on a NIKON N-STORM microscope on a Ti-E stand equipped with a Nikon 100x 1.49 NA TIRF oil objective, Perfect Focus System, Agilent MLC400 laser bed with 405 nm (50 mW*), 488 nm (80 mW*), 561 nm (80 mW*) and 640 nm (125 mW*) solid-state lasers and Andor iXon Ultra EM-CCD camera as previously described[27]. Samples were maintained in an OKO environmental chamber at 28^{o}C for maximum system stability during imaging. All dSTORM experiments were performed in TIRF mode on a NIKON N-STORM microscope as described previously[27]. During dSTORM acquisition, the sample was continuously illuminated at 640 nm for 20,000 frames (40 x 40 μm, 9.2 ms exposure time) and the 405 nm laser was used for back pumping. Samples were imaged in switching buffer (0.5 mg/ml glucose oxidase, 40 μg/ml catalase, 10% (w/v) glucose and 100 mM β-mercaptoethylamine in PBS, pH 7.4) to induce Alexa 647 blinking[28]. The 20,000 frames were captured with Nikon NIS ELEMENTS v4.5 and reconstructed in ThunderSTORM imageJ plugin[29] where approximate molecule positions are determined. The settings used were Gaussian PSF modelling and maximum likelihood fitting. Further post processing analysis include: lateral drift correction, local density filtering where the distance ratio was 50 and minimum number of neighbors was 5 and merging of reappearing localizations. All detections in sequential within 20 nm were merged. Gaussian rendering was

implemented for visualization. Points in the reconstructed images represent individually identified fluorescent blinking events, which are referred to as detections.

Cluster analysis

After localising detections, density-based spatial clustering of applications with noise (DBSCAN) [30] was used to group detections into clusters and segment clusters of arbitrary shape such as on collagen fibres. For DBSCAN the radius of the local neighbourhood was set to 50 nm and the minimum number of directly reachable points was set to 10. Edge points were included in clusters. Cluster area was calculated by placing a circle with a radius of 50 nm over every detection in a cluster and calculating the union of these circles. This was estimated using a grid with a pixel size of 5 nm and image based dilation. Cluster density was defined as the number of detections within a cluster divided by the cluster area. This analysis was performed on whole fields of view using the open-source software KNIME[25]. DBSCAN was implemented within KNIME using the R package 'dbscan'.

Statistical analysis

Each experiment was performed at least three times and results are shown as mean± SEM. Data were analysed using PRISM v7.04 (GraphPad, San Diego, CA.) and statistical analysis was by one-way ANOVA with a Bonferroni *post-hoc* test. Significance was set at $p \leq 0.05$.

Results

The effect of NECA and inhibitors of feedback agonists on aggregation and secretion induced by collagen.

We investigated the effect of the A_{2A} receptor agonist NECA[31] (1, 10 and 100 μM) on platelet aggregation induced by low (2 μg/ml) and intermediate (10 μg/ml) concentrations of collagen (Supplementary Fig. 1). Both concentrations of collagen stimulated aggregation after a delay

of approximately 15 sec which reached approximately 95% of the maximal aggregation within 2 min (Supplementary Fig. 1). In the presence of NECA (1-100 μM), aggregation to a low concentration of collagen (2 μg/ml) was partially reduced and began to slowly return to the resting level. In contrast, NECA (1 μM) had no significant effect on the response to an intermediate concentration (10 μg/ml) of collagen whereas the maximal level of aggregation, but not the time course of response, was partially inhibited in the presence of NECA (10 and 100 μM). For these reasons, 100 μM NECA was chosen for further experiments.

To investigate whether the mechanism of inhibition of the response to collagen by NECA was due to inhibition of the action of the secondary agonists, ADP and thromboxane A_2 (TxA_2), we first monitored aggregation and dense granule secretion (ATP release) in the presence of maximally effective concentrations of the $P2Y_{12}$ receptor antagonist, cangrelor (10 μM) and the cyclooxygenase inhibitor indomethacin (10 μM). Both agents caused a partial inhibition of aggregation to collagen (10 μg/ml) which was similar to that induced by NECA (100 μM) but neither agent, separately or in combination with NECA had an effect on ATP secretion (Supplementary Fig. 2). A slightly greater level of inhibition of secretion was seen when cangrelor and indomethacin were used in combination, or when given with NECA, which did reach statistical significance (Fig. 1A). All three inhibitors blocked aggregation and secretion to a lower concentration of collagen (2 μg/ml) (Fig. 1B). The lack of an additional effect of NECA in the presence of cangrelor and indomethacin on aggregation and secretion to the higher concentration of collagen suggests that its inhibitory action is mediated predominantly by inhibition of the secondary feedback agonists.

One explanation for the relatively weak effect of NECA on aggregation and secretion to collagen (10 μg/ml) is that it induces only a small increase in cAMP. To address this, we used forskolin which induces powerful activation of adenylyl cyclase and therefore a much larger increase in cAMP (Supplementary Fig. 3A). Forskolin induced a similar effect to the

combination of cangrelor and indomethacin on collagen induced aggregation and ATP secretion (Fig. 1C). The combination of all three agents caused a slightly greater level of inhibition of aggregation and ATP secretion to that seen with NECA, cangrelor and indomethacin.

To investigate whether the inhibitory effect of NECA was due to blockade of signalling by GPVI, we investigated the effect on aggregation and secretion induced by the GPVI-specific ligand, CRP. NECA or the combination of indomethacin and cangrelor has no significant effect on platelet aggregation to a high concentration of CRP (10 µg/ml), although the combination of indomethacin and cangrelor partially reduced secretion (Fig. 2A). Surprisingly however, both responses were markedly inhibited in the presence of all three inhibitors. In contrast, NECA alone and the combination of indomethacin and cangrelor blocked the response to a lower concentration of CRP (1 µg/ml) (Fig. 2B). This demonstrates that NECA is able to inhibit aggregation and secretion to CRP through a pathway that is unmasked by the absence of signalling by secondary agonists.

We extended the studies on secretion to CRP (10 µg/ml) to measure the α-granule marker P-selectin (Supplementary Fig 3B). CRP causes a significant increase in P-selectin exposure which is reduced in the presence of NECA and forskolin.

Together these results show that NECA and forskolin block the response to threshold activation of GPVI in the presence of inhibition of the feedback agonists TxA_2 and ADP. The inhibitory effect of NECA is largely overcome at higher concentrations of collagen but not CRP.

The effect of NECA and forskolin on collagen induced protein tyrosine phosphorylation

To investigate the mechanism underlying the inhibitory effect of the adenosine A_{2A} receptor agonist NECA, we measured phosphorylation of key signalling proteins in the GPVI pathway by western blotting using phosphospecific antibodies. We chose to focus on three proteins that

are phosphorylated at multiple sites and which play key roles in GPVI signalling, namely Syk, LAT and PLCγ2. We elected to use forskolin in the phosphorylation studies because it induces a greater increase in cAMP (Supplementary Fig. 3A). Phosphorylation was measured in the presence of maximally-effective concentrations of indomethacin and apyrase, inhibitors of the TxA_2 and ADP feedback pathways, respectively. Incubation times used are in-line with previously published work[18, 32]. Strikingly, forskolin caused a minimal reduction in tyrosine phosphorylation of Syk at Y323 (docking), Y352 (docking) and Y525/526 (activation), LAT at Y132 (docking) and Y200 (docking), and PLCγ2 at Y759 (activation and docking) and Y1217 (activation) (Fig. 3ii). Similarly, NECA had no significant effect on phosphorylation of Syk, LAT and PLCγ2 at Y525/526, Y200 and Y1217, respectively (Supplementary Fig. 4). These results demonstrate that neither elevation of cAMP via stimulation of the adenosine A_{2A} receptor or using the adenylyl cyclase activator, forskolin had a major effect on tyrosine kinase signalling downstream of platelet activation by collagen.

Adenosine and forskolin have no effect on platelet spreading or Ca^{2+} elevation induced by collagen

The adhesion of platelets to a collagen surface leads to a rapid reorganization of the actin cytoskeleton resulting in formation of filopodia and eventually lamellipodia and stress fibres. Addition of NECA or forskolin to platelets that had been incubated a collagen-coated surface for 30 min had no significant effect on the degree of adhesion or platelet morphology as shown by measurement of surface area (Fig. 4A). A similar result was seen when NECA and forskolin were given prior to the onset of spreading (Fig. 4B) although the degree of platelet adhesion was markedly reduced. This suggests that adenosine and forskolin have an inhibitory effect prior to spreading but this is lost once platelet spreading and collagen signalling has begun.

To investigate if cAMP is inhibiting Ca^{2+} mobilization following collagen stimulation, we measured the frequency and fluorescence intensity of Ca^{2+} spikes in platelets spread on a collagen surface following treatment with forskolin, indomethacin and cangrelor. We elected to use forskolin in the single cell Ca^{2+} studies because it induces a greater increase in cAMP (Supplementary Fig. 3A). Forskolin either alone or in combination with indomethacin and cangrelor had no effect on the frequency of Ca^{2+} spikes or the fluorescence intensity of these spikes (Fig. 5). This suggests that Ca^{2+} elevation induced by collagen is not affected by elevation of cAMP.

NECA and forskolin do not change GPVI receptor clustering

To determine whether NECA or forskolin had an effect on endogenous GPVI clusters which form in platelets spreading on immobilized collagen, single molecule super resolution microscopy (dSTORM) was used. GPVI was labelled using a Fab fragment of the monoclonal antibody to pan-GPVI, 1G5[23]. The platelets were allowed to adhere to collagen for 30 min before being treated with NECA or forskolin for a further 30 min to determine whether GPVI distribution was affected. Diffraction limited TIRF microscopy showed a characteristic GPVI distribution with enrichment of GPVI along collagen fibres, as has been shown previously[27]. dSTORM images show the localization of single molecule detections and the DBSCAN cluster plot shows clustering of the detections in the image where different clusters are represented by different colors. The largest clusters of GPVI can be seen along the collagen fibres. Results from the quantitative cluster analysis (Fig. 6Bi-iv) show that neither NECA nor forskolin altered GPVI clustering, with the number of clusters, number of detections per cluster and cluster area and density all similar to control values.

NECA and forskolin inhibit TxA_2 induced platelet aggregation

The effect of NECA and forskolin on TxB_2 formation and platelet aggregation induced by the TxA_2 mimetic, U46619, was investigated to determine the basis of loss of platelet activation. The thromboxane A_2 (TP) receptor agonist U46619 caused ~60% maximal platelet aggregation. However, both NECA and forskolin caused a significant reduction in U46619 induced platelet aggregation (Fig. 7A) with NECA reducing aggregation to ~10% and forskolin causing complete blockade. This suggests that cAMP is inhibiting thromboxane receptor signalling. In addition both NECA and forskolin also partially decreased TxB_2 formation by collagen (Supplementary Fig. 3C) demonstrating that elevation of cAMP causes inhibition both upstream and downstream of TxA_2.

ADP does not cause aggregation in washed platelets due to desensitisation of the $P2Y_1$ receptor which prevents synergy with the $P2Y_{12}$ receptor. To investigate the effect of NECA on the response to $P2Y_{12}$ receptor activation, we monitored platelet aggregation induced by a low concentration of collagen (2 μg/ml) in the absence and presence of ADP (Fig. 7C&D). On its own, ADP had no effect on aggregation. NECA inhibited the response to the low dose of collagen as described in Fig.1, but had no effect on the response to collagen in the presence of ADP (Fig. 7C&D). This demonstrates that NECA has a greater effect on the TxA_2 arm of the feedback agonists.

Discussion

In the present study, we show that signalling via the adenosine A_{2A} receptor and stimulation of adenylyl cyclase blocked platelet aggregation and ATP secretion induced by low concentrations of collagen but only had a partial effect against higher concentrations and present evidence that this is mediated in part by inhibition of TxA_2 receptor signalling. Adenosine also blocked platelet aggregation and ATP secretion by a low concentration of the GPVI-selective agonist CRP, but only inhibited the response to a higher concentration in the

presence of the combination of a $P2Y_{12}$ antagonist and cyclooxygenase inhibitor. Together these results show that platelet activation induced by high concentrations of collagen and the GPVI-specific CRP is relatively refractory to the inhibitory adenosine A_{2A} receptor unless inhibitors of the feedback agonists ADP and TxA_2 are also present.

The observation that adenosine or the more powerful inhibitor forskolin, which induces a greater increase in cAMP, had no effect on clustering of GPVI, tyrosine phosphorylation of Syk, LAT and PLCγ2, elevation of Ca^{2+} and spreading of platelets on collagen demonstrates that elevation of cAMP has a minimal effect on the proximal signalling events by GPVI. This therefore explains why collagen in able to initiate platelet activation at sites of lesion in the vessel wall despite the increase in cAMP. The inhibitory effect of NECA against lower concentrations of collagen is therefore mediated downstream of Ca^{2+} signalling, including inhibition of the formation and action of TxA_2 and secretion of ADP as shown.

Loyau et al[21] have proposed that elevation of cAMP maintains GPVI in a monomeric form as shown using the mAb 9E18 which recognises dimeric but not monomeric GPVI. This suggests that elevation of cAMP should induce powerful inhibition of platelet activation by collagen as this binds selectively to the dimeric form of GPVI[12]. The observation that the proximal events in GPVI signalling are not altered by NECA or by forskolin therefore suggests the effect of cAMP on 9E18 binding may not be directly related to GPVI/FcRγ-dependent signalling. Takayama et al[22] reported that elevation of cAMP leads to internalisation of GPVI in human platelets, but the lack of effect on signalling indicates that this is a relatively slow event. Taken together, our data suggests that the inhibitory effect of cAMP occurs downstream of GPVI signalling, including inhibition of the feedback agonist TxA_2 and possibly via additional effects on integrin αIIbβ3 activation and secretion[3, 33, 34].

Adenosine has been previously reported to inhibit platelet activation by a number of G protein-coupled receptor agonists including thrombin, ADP and TxA_2[13, 14, 31, 35]. In all cases, inhibition was mediated by loss of PLCβ activation in combination with elevation of cGMP and inhibition of IP_3-induced Ca^{2+} release via the PKG-IRAG-IP_3 receptor complex. In our study, single cell Ca^{2+} measurements during collagen induced signalling in the presence of forskolin however revealed no effect on Ca^{2+} release signalling, questioning the significance of this mechanism.

In conclusion, the observation that the proximal events in collagen-mediated signalling including Ca^{2+} flux are not altered by elevation of cAMP with adenosine or forskolin is consistent with a critical role for collagen/GPVI in mediating platelet activation at sites of injury to the vasculature, despite the presence of the cAMP-elevating agent, prostacyclin. Inhibition of platelet activation by adenosine and forskolin is overcome at higher concentrations of collagen which mimics the situation in the vessel wall. In addition, these results may have important implications for the therapeutic action of the $P2Y_{12}$ inhibitor ticagrelor which also inhibits adenosine uptake, and this would further inhibit platelet signalling notably by low concentrations of collagen. Similar interactions may benefit the therapeutic action of the clinically-used PDE inhibitors such as cilostazol and dipyridamole.

Authors' Contributions: J. C. Clark has performed experiments, analysed data, wrote and edited manuscript. S. Watson performed experiments and analysed data. D. M. Kavanagh provided training in super resolution microscopy experiments and expertise for the study design and cluster analysis. J. A. Pike has analysed data and developed the programmes for image and cluster analysis. N. S. Poulter provided expertise for super resolution microscopy experiment study design and cluster analysis. R. K. Andrews and E. E. Gardiner provided key materials. S. J. Hill provided supervision, concept, funding and contributed to manuscript editing. S. P. Watson provided supervision, funding, study design and concept, reviewed data, wrote and edited manuscript. All authors have read the manuscript.

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Disclosure of Conflict of Interests: The authors declare no conflict of interest

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Figure Captions

Fig. 1 The effect of NECA, forskolin, indomethacin (Indo) and cangrelor (Cang) on collagen induced platelet aggregation and ATP secretion. Platelet aggregation induced by (A, C) intermediate (10 μg/ml) and (B) low (2 μg/ml) concentrations of collagen was monitored by light transmission aggregometry at 37°C with constant stirring at 1200 rpm. Secretion of ATP was measured using luciferin-luciferase Chromo-lume reagent. The effect of NECA (100 μM), indomethacin (10 μM) and cangrelor (10 μM) on (Ai) intermediate (10 μg/ml) and (Bi) low (2 μg/ml) concentration collagen induced aggregation. The effect of NECA (100 μM), indomethacin (10 μM) and cangrelor (10 μM) on (Aii) intermediate (10 μg/ml) and (Bii) low (2 μg/ml) concentration collagen induced ATP secretion. Representative (Aiii) aggregation and (Aiv) secretion traces showing the effect of the inhibitors on intermediate concentration (10 μg/ml) collagen induced platelet aggregation and ATP secretion. Representative (Biii) aggregation and (Biv) secretion traces showing the effect of the inhibitors on low concentration (2 μg/ml) collagen induced platelet aggregation and ATP secretion. (C) The effect of forskolin (10 μM), indomethacin (10 μM) and cangrelor (10 μM) on intermediate (10 μg/ml) concentration collagen induced aggregation and ATP secretion. Representative (Ciii) aggregation and (Civ) secretion traces showing the effect of the inhibitors on intermediate concentration (10 μg/ml) collagen induced platelet aggregation and ATP secretion. Significance was measured using one-way ANOVA with a Bonferroni post-hoc test where, $P<0.05$. Data presented as mean±SEM (n=6).

Fig. 2 The effect of NECA, indomethacin (Indo) and cangrelor (Cang) on collagen-related-peptide (CRP) induced platelet aggregation and ATP secretion. Platelet aggregation induced by (A) high (10 μg/ml) and (B) low (1 μg/ml) concentrations of CRP was monitored by light transmission aggregometry at 37°C with constant stirring at 1200 rpm. Secretion of ATP was measured using luciferin-luciferase Chromo-lume reagent. The effect of NECA (100 μM), indomethacin (10 μM) and cangrelor (10 μM) on (Ai) high (10 μg/ml) and (Bi) low (1 μg/ml) concentration CRP induced aggregation. The effect of NECA (100 μM), indomethacin (10 μM) and cangrelor (10 μM) on (Aii) high (10 μg/ml) and (Bii) low (1 μg/ml) concentration CRP induced ATP secretion. Representative (Aiii) aggregation and (Aiv) secretion traces showing the effect of the inhibitors on high concentration

(10 μg/ml) CRP induced platelet aggregation and ATP secretion. Representative (Biii) aggregation and (Biv) secretion traces showing the effect of the inhibitors on low (1 μg/ml) concentration CRP induced platelet aggregation and ATP secretion. Significance was measured using one-way ANOVA with a Bonferroni post-hoc test where: *P<0.05.* Data presented as mean±SEM (n=3-6).

Fig. 3 The effect of forskolin on collagen induced PLCγ2, Syk and LAT phosphorylation. The effect of forskolin (10 μM) in the presence of maximally-effective concentrations of indomethacin (10 μM) and apyrase (10 μM) on collagen induced (10 μg/ml) phosphorylation investigated by western blotting with (i) the phosphotyrosine antibody 4G10 and (ii) phosphospecific antibodies to PLCγ2, Syk and LAT. (iii) Quantification of band intensities where the blue line represents control and the red line represents phosphorylation in the presence of forskolin. Data presented as mean±SEM (n=5).

Fig. 4 NECA and forskolin have no significant effect on platelet spreading on collagen. (A) Washed platelets ($2x10^7$/ml) were spread on a collagen-coated surface for 30 min at 37°C and then NECA (100 μM) and forskolin (10 μM) were added and platelets spread for an additional 30 min. Five fields of view per treatment per experiment were captured using epifluorescence microscopy and analysed with KMINE software (Field of view = 211 μm x 211 μm). (Ai) Representative zoomed-in images of platelet spreading with the indicated treatments. (Field of view= 94 μm x 94 μm) (Scale bar: 5 μm). Quantification of (Aii) surface area and (Aiii) platelet adhesion. (B) Washed platelets ($2x10^7$/ml) were pre-incubated with NECA (100 μM) and forskolin (10 μM) for 30 min followed by spreading on a collagen-coated surface for 30 min at 37°C. Five fields of view per treatment per experiment were captured using epifluorescence microscopy and analysed with KMINE software (Field of view = 211 μm x 211 μm). (Bi) Representative zoomed-in images of platelet spreading with the indicated treatments. (Field of view= 94 μm x 94 μm) (Scale bar: 5 μm). Quantification of (Bii) surface area and (Biii) platelet adhesion. Significance was measured using one-way ANOVA with a Bonferroni post-hoc test where: *P<0.05.* Data presented as mean±SEM (n=3).

Fig. 5 Forskolin, indomethacin and cangrelor have no significant effect on collagen induced Ca^{2+} mobilisation. Washed platelets were loaded with Oregon green 488 BAPTA-1-AM and diluted to $2x10^7$/ml. Prior to spreading the platelets were incubated with forskolin (10 μM) or indomethacin (10

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Fig. 6 NECA and forskolin do not change GPVI receptor clustering on collagen. Washed platelets ($2x10^7$/ml) were incubated with 1G5-Fab and allowed to spread on collagen for 30 min at 37°C. NECA (100 μM) and forskolin (10 μM) were then added to the platelet suspensions and platelets were spread for an additional 30 min. All STORM experiments were performed on a NIKON N-STORM microscope. Seven fields of view per treatment per experiment were captured and the 20,000 frames captured with Nikon NIS ELEMENTS v4.5 were reconstructed in ThunderSTORM imageJ plugin. Cluster analysis was performed in KMINE software with an algorithm based on DBSCAN with an additional setting based on image dilation. (A) Representative TIRF and reconstructed STORM images of platelet spreading and detections with the indicated treatments. Cluster plots produced in MATLAB show the clusters, each represented by a different arbitrary colour. (Field of view = 40 x 40 μm) (scale bar: 2 μm). (B) Relative quantification of (i) number of clusters, (ii) number of detections per cluster and (iii) cluster area and (iv) density from seven fields of view per treatment per experiment. Data presented as mean±SEM (n=3).

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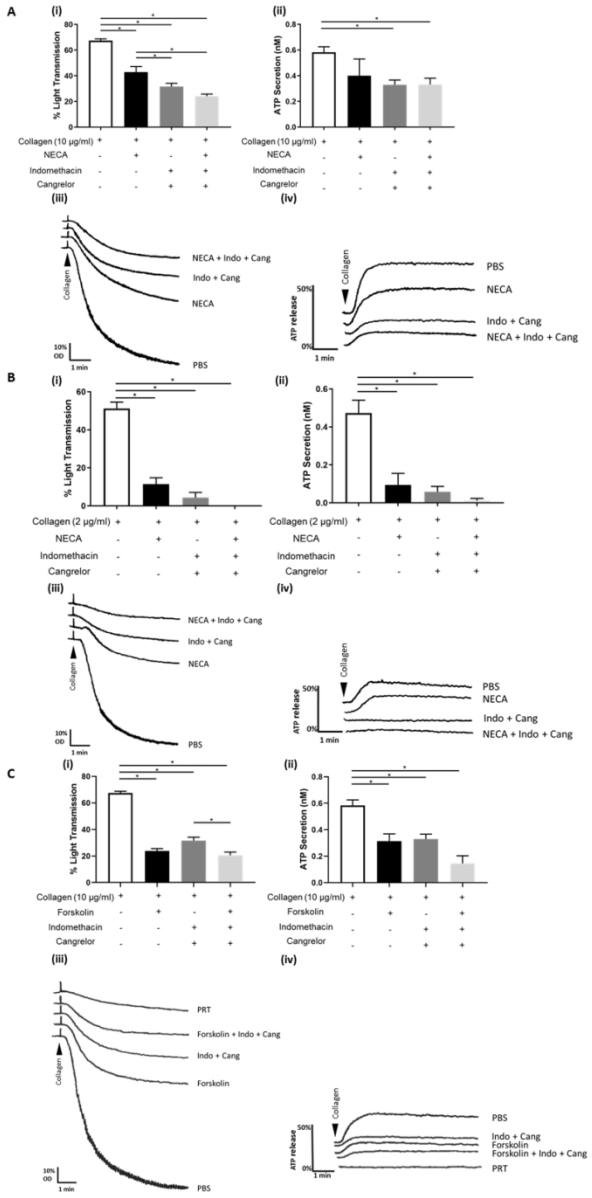

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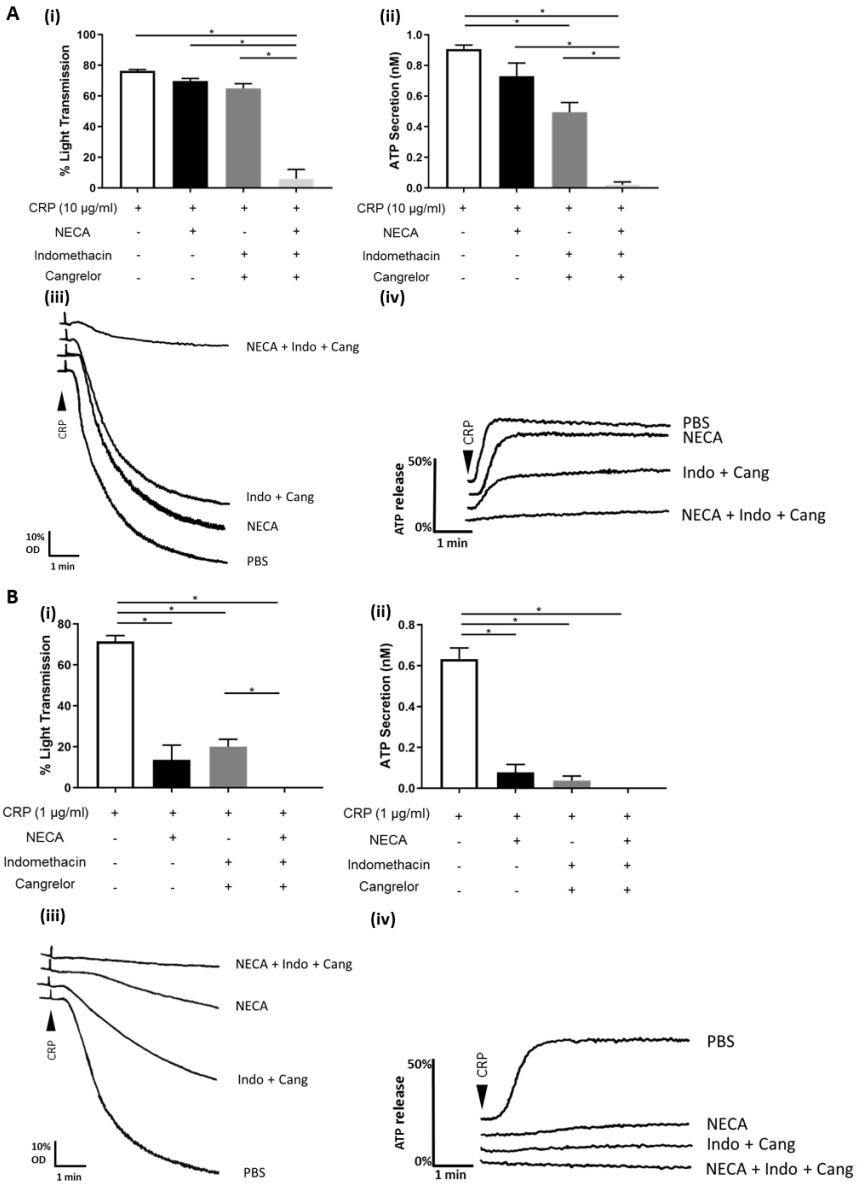

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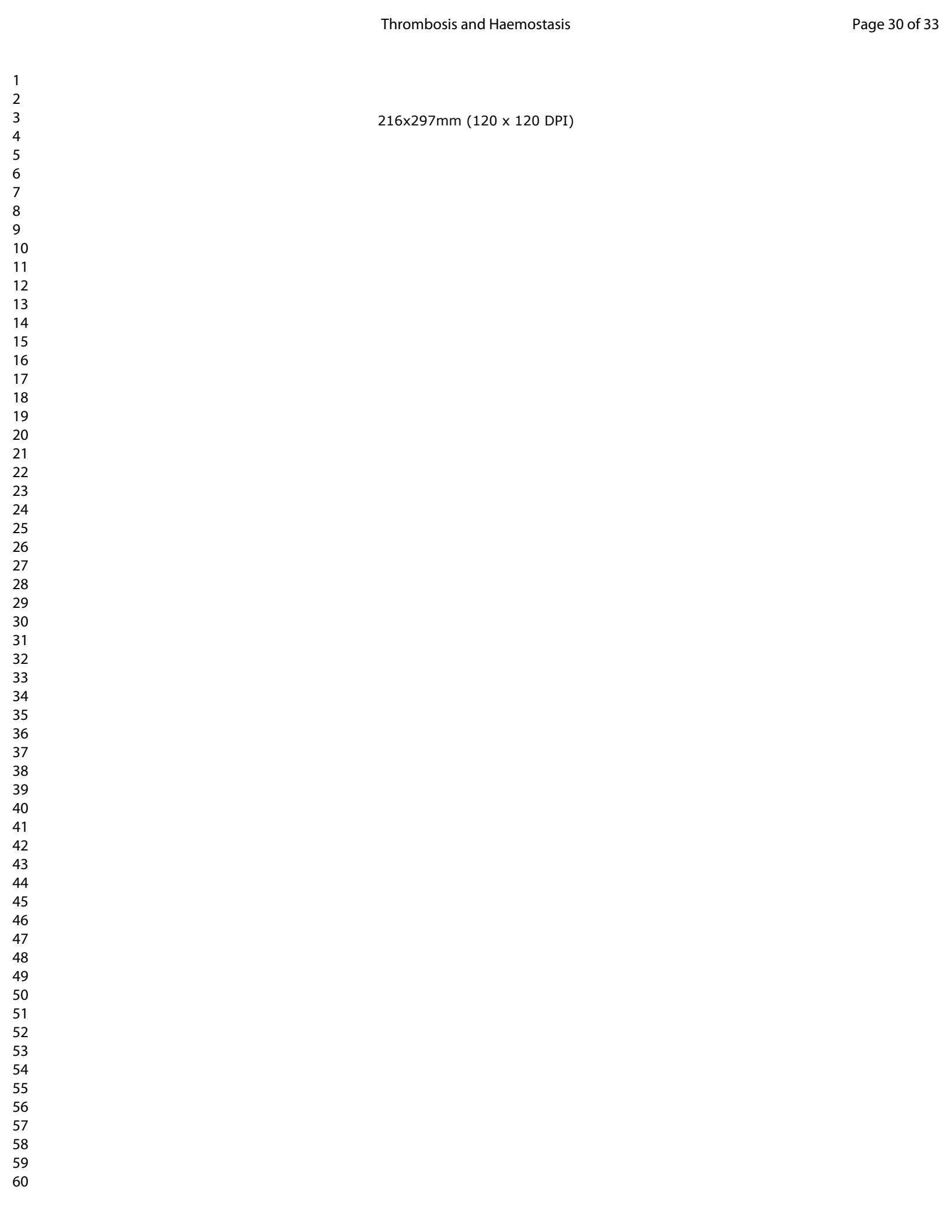
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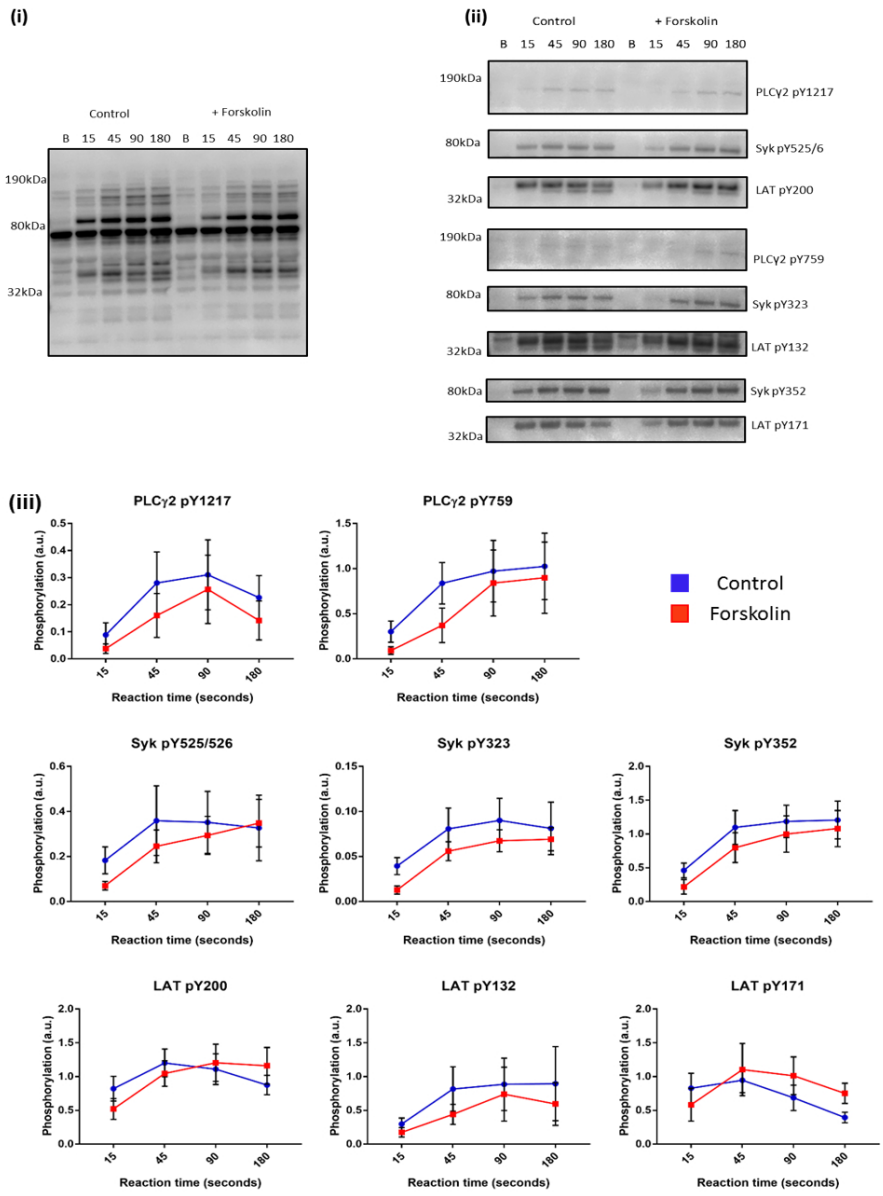

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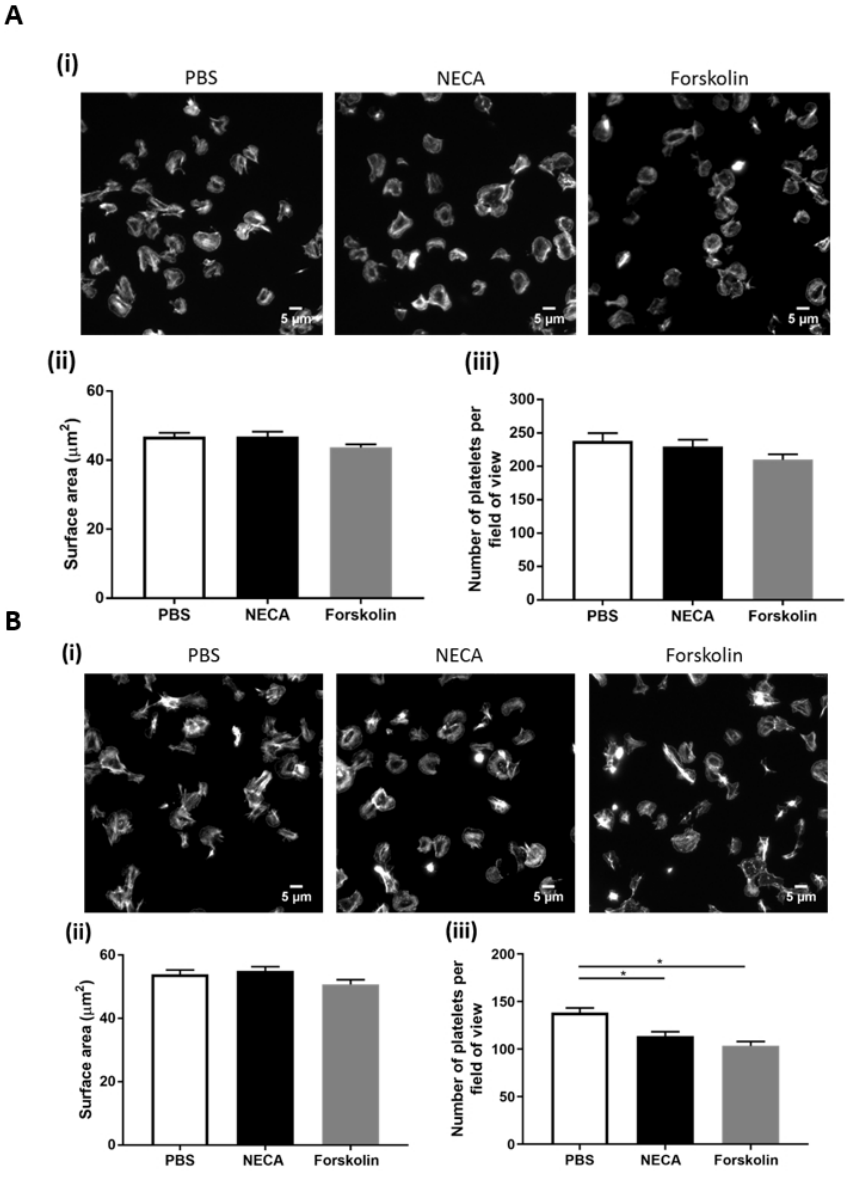

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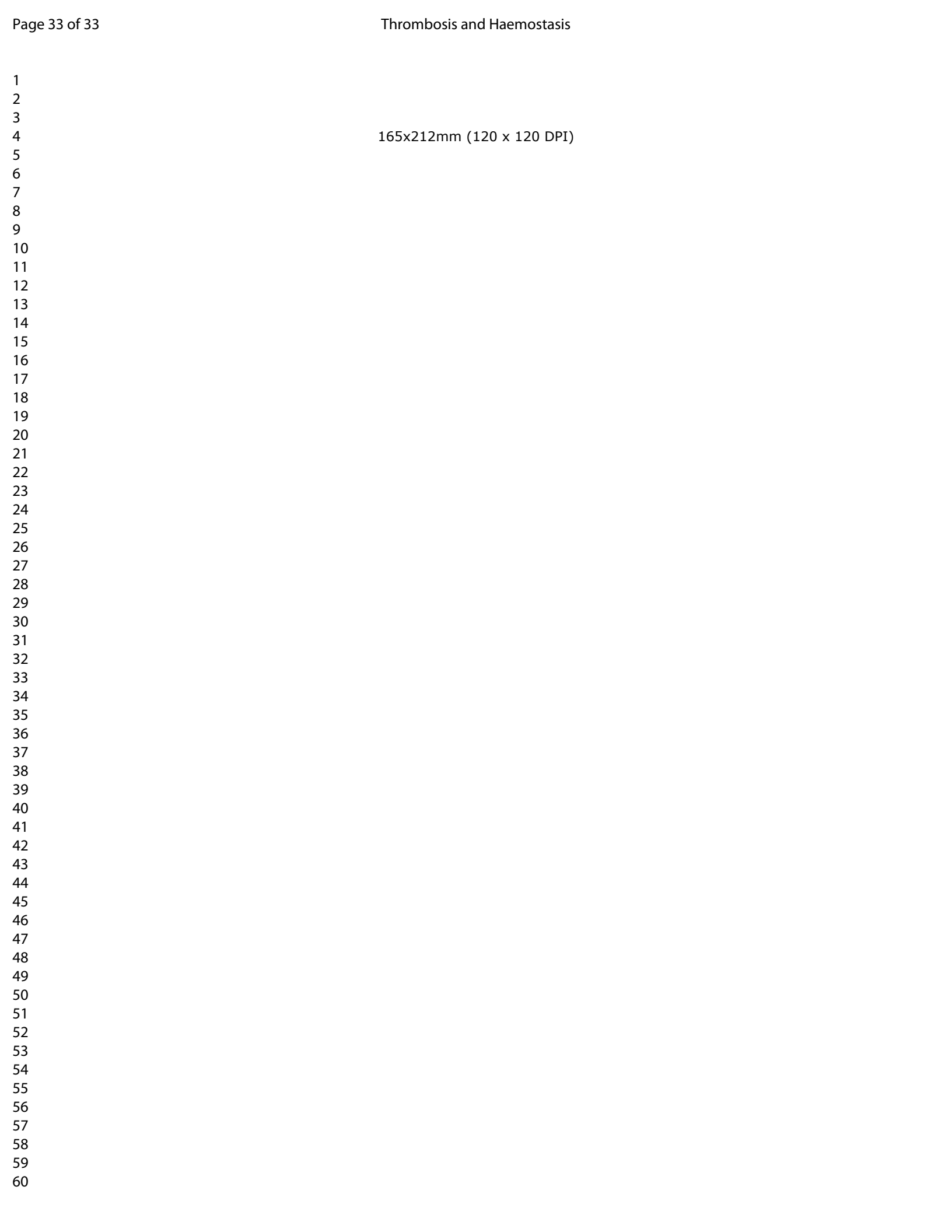
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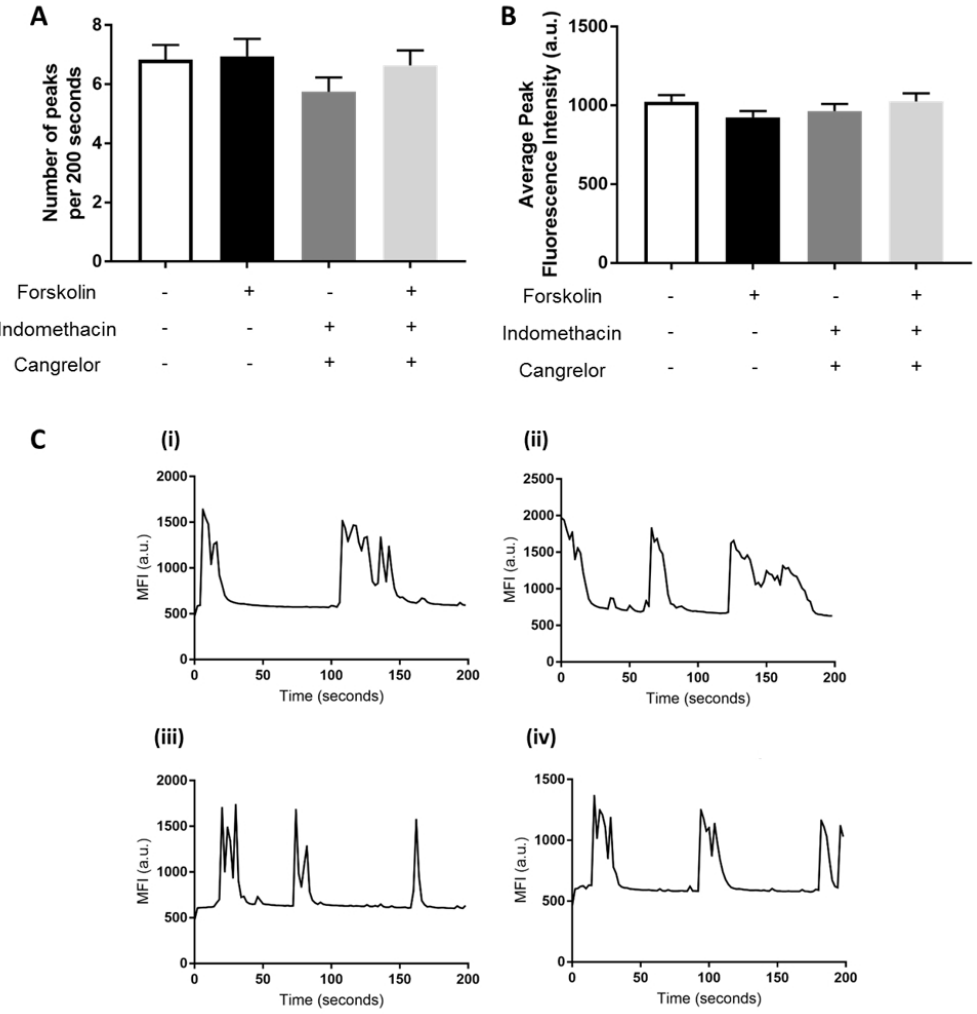

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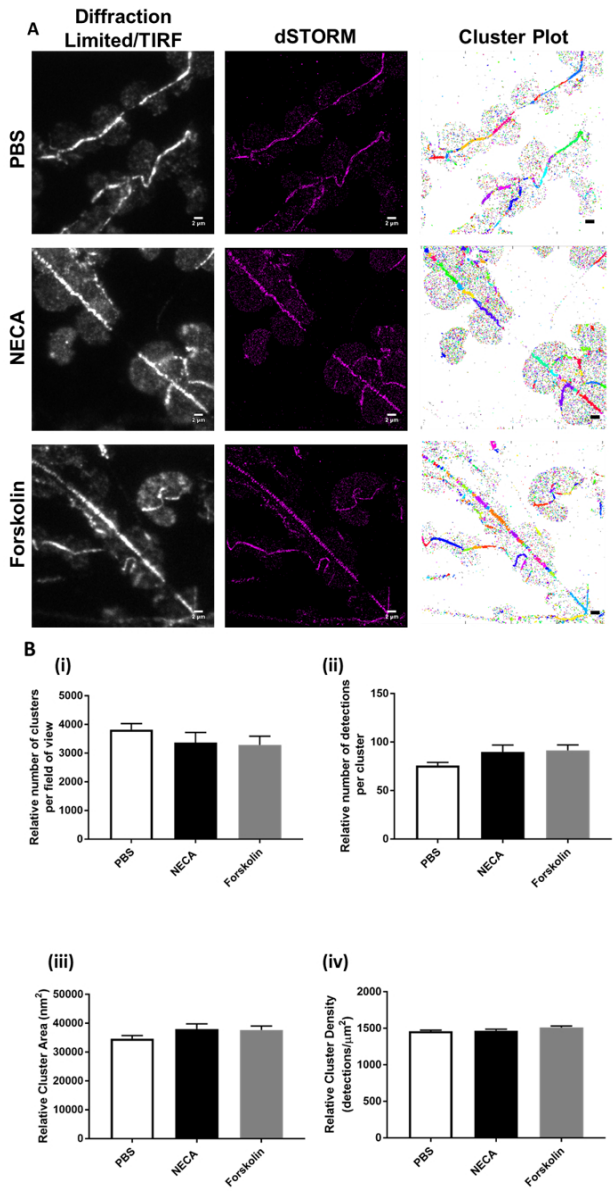

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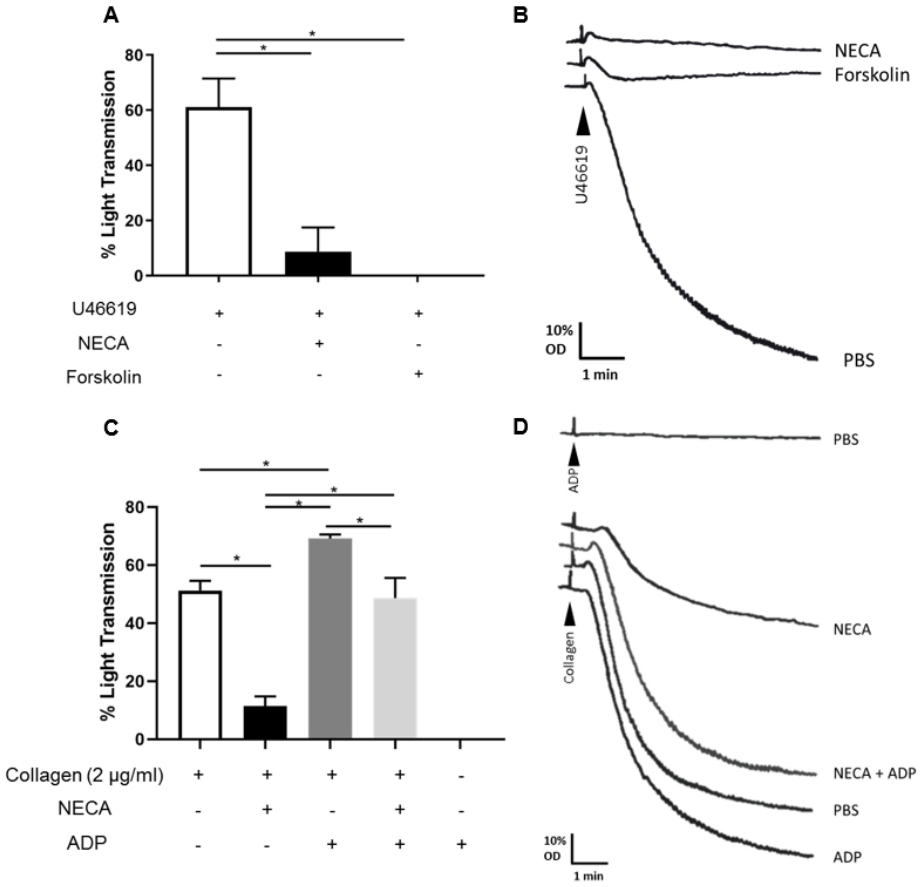

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