Pharmacological Characterization of Calcium-Activated Potassium Channel Function in Human Platelets

by

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ABSTRACT

Background: Platelets and endothelial cells play an important role in maintaining vascular tone and hemostasis, critical to maintaining the health of the cardiovascular system. In resistance arteries, stimulation of endothelial cell small (SK_{Ca}) and intermediate (IK_{Ca}) conductance calcium-activated potassium channels provides a negative-feedback mechanism to limit agonist-induced vasoconstriction. Additionally, endothelial cell K_{Ca} channels in conjunction with nitric oxide (NO) mediate vasodilation in response to agonists and physical stimuli. Platelets, like endothelial cells, possess K_{Ca} channels and generate NO via endothelial nitric oxide synthase (eNOS). NO is known to limit platelet aggregation but the role of K_{Ca} channels in platelet function and NO-generation has not yet been explored. It was hypothesized that activation of K_{Ca} channels inhibits platelet aggregation and enhances platelet NO production. The objective was to pharmacologically characterize SK_{Ca} and IK_{Ca} channel function in platelets, and investigate their role in platelet NO production.

Methods: Blood was collected from healthy human volunteers following ethics approval. The platelets were then isolated from the collected blood, according to standard protocol. Immunofluorescence microscopy was used to determine K_{Ca} channel subtypes present within platelets, and their localization within the platelet. Aggregometry was performed in the presence of IK_{Ca} (SKA-31) and SK_{Ca} (CyPPA) channel activators, and IK_{Ca} (TRAM-34) and SK_{Ca} (apamin) channel blockers. A novel flow chamber model, the Q-Sense Quartz Crystal Microbalance (QCM), along with confocal microscopy was used to investigate platelet adhesion and aggregation under flow conditions in response to K_{Ca} channel activators. Flow cytometry of DAF-FM stained platelets was used to measure changes in NO generation. Calcium signaling was measured using flow cytometry of Fluo-4 AM stained platelets at different time points of aggregation in response

to K_{Ca} channel activators. ATP and P-selectin secretion were measured as markers of dense and alpha granule secretion, respectively.

Results: Confocal microscopy of platelets demonstrated the presence of IK_{Ca} channels in platelets, with the presence of SK_{Ca} channels in some individuals. IK_{Ca} channels were localized to the cytosolic portion of the platelet plasma membrane. Activation of IK_{Ca} channel with SKA-31, or SK_{Ca} channel activation with CyPPA demonstrated concentration-dependent inhibition of collagen-induced aggregation. IK_{Ca} selective channel blocker, TRAM-34, reversed the anti-aggregatory effects of SKA-31 but not CyPPA. SK_{Ca} channel-selective blocker, apamin, did not reverse the effect of either SKA-31 or CyPPA, but weakly inhibited platelet aggregation alone. The QCM demonstrated that SKA-31 inhibits platelet aggregation under laminar flow conditions, but CyPPA does not. Surprisingly, SKA-31 and CyPPA treatment inhibited platelet-NO generation. Calcium signaling flow cytometry demonstrated a significant inhibition of calcium flow during aggregation with SKA-31 treatment, but not CyPPA. Further investigation demonstrated SKA-31 inhibiting dense and alpha granule secretion, whereas CyPPA only inhibited dense granule secretion.

Conclusions: These results suggest that IK_{Ca} may be the dominant K_{Ca} channel within platelets, which upon pharmacological activation inhibits platelet aggregation. IK_{Ca} channel activation also inhibits platelet NO generation, calcium signaling, as well as dense and alpha granule secretion within platelets. Thus IK_{Ca} channels may provide a novel therapeutic target to inhibit platelet aggregation.

PREFACE

This thesis is an original work by Valentina Helene Back. The research project, of which this thesis is a part, received research ethics approval for human platelet isolation from the University of Alberta Research Ethics Board for Dr. Jurasz's lab, Project name "NOS-based platelet subpopulations", No. Pro00029836, March 8, 2012.

This work is done in collaboration with Dr. F. Plane, based on her work on K_{Ca} channels in endothelial cells. Through the collaboration with Dr. F. Plane, the K_{Ca} channel antibodies and blocking peptides were obtained through Dr. S. Sandow from Australia. My colleague, G. Lesyk was a vital component in teaching of the Q-sense™ Quartz Crystal Microbalance apparatus, and microscopy, the results of which are seen in Section 3.5.

No part of this thesis has previously been published.

DEDICATION

Dedicated to all the people who helped, supported, and believed in me along the way.

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First, a big thank you to Dr. Paul Jurasz, for being so kind and giving me this opportunity by taking me into your lab. I have continuously learned throughout, and I will always remember my time here.

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ABBREVIATIONS

AMR – Ashwell-Morrell receptor

ANOVA – analysis of variance

BH_4 – (6R)-5,6,7,8-tetrahydro-L-biopterin

BK_{Ca} – large conductance calcium activated potassium channels

Ca^{2+} – calcium ions

COX – cyclo-oxygenase

DAG – diacylglycerol

DMS – demarcation membrane system

DMSO – dimethyl sulfoxide

DTS – dense tubular system

ECM – extracellular matrix

EDRF – endothelium-derived relaxation factor

EDTA – ethylenediaminetetraacetate

eNOS – endothelial nitric oxide synthase

ER – endoplasmic reticulum

FAD – flavin adenine dinucleotide

FMN – flavin mononucleotide

GP – glycoprotein

GPCR – G-protein coupled receptors

IK_{Ca} – intermediate conductance calcium activated potassium channels

iNOS – inducible nitric oxide synthase

IP_3 – inositol triphosphate

IRAG – inositol-1,4,5-triphosphate receptor-associated cGMP kinase substrate

K^+ – potassium ions

K_{Ca} – calcium activated potassium channels

MI – myocardial infarction

MMP-2 – matrix metalloproteinase-2

NAADP – nicotinic acid adenine dinucleotide phosphate

NADPH – nicotinamide-adenine-dinucleotide phosphate

nNOS – neuronal nitric oxide synthase

NO – nitric oxide

PBS – phosphate buffered saline

PGI_2 – prostacyclin

PIP_2 – phosphatidylinositol 4,5 bisphosphate

PKA – protein kinase A

PKC – protein kinase C

PLC – phospholipase C

PRP – platelet rich plasma

PPP – platelet poor plasma

PS – phosphatidyl serine

QCM – quartz crystal microbalance

RM – repeated measures

ROC – receptor operated calcium channel

SK_{Ca} – small conductance calcium activated potassium channels

SMOC – second messenger operated calcium channel

SNARE – soluble N-ethylmaleimide-sensitive factor attachment protein receptor

SOC – store operated calcium channel

STIM1 – stromal interaction molecule 1

TRAP – thrombin receptor-activating peptide

TPC – two-pore channels

TPO – thrombopoietin

TRAP – thrombin receptor-activating peptide

TRPC – transient receptor potential channels

TXA_2 – thromboxane A_2

VAMPs – vesicle-SNAREs

VASP – vasodilator stimulated phosphoprotein

VSM – vascular smooth muscle

vWF – von Willebrand factor

1. INTRODUCTION

1.1 Platelet Biology

1.1.1 Cardiovascular Disease

Cardiovascular disease encompasses a milieu of syndromes and diseases affecting the cardiovascular system, including atherosclerosis, myocardial infarction, stroke, heart failure, and arrhythmia. Cardiovascular diseases are responsible for approximately one quarter of deaths yearly in Americans.[1] In Canada, ischemic stroke and myocardial infarction are responsible for nearly half of all cardiovascular deaths.[2] Because of its complexity, cardiovascular disease involves factors such as endothelial dysfunction, increased blood pressure, and a decrease in nitric oxide generation, all of which contribute to an increased reactivity in platelets. Since platelets can form occlusive aggregates or thrombi thereby resulting in blockage of a blood vessel, they play a major role in ischemic diseases. Currently many treatment options are available that target platelet enzymes or receptors to inhibit platelet function, but despite these options the incidence of thrombosis remains high. There are currently no treatment options that targets any ion channel within platelets. Due to the involvement of platelets in thrombosis and ischemic diseases, there is a necessity to further study platelet function and increase understanding of platelet biology, in order to identify novel targets.

1.1.2 Platelet Structure

Platelets, also referred to as thrombocytes, are small, anucleate, megakaryocyte-derived cell fragments. Platelets are approximately 2-4μm in diameter, with a volume of about 6fl. Their primary physiological function is the maintenance of hemostasis, the physiological formation of a platelet plug, also called an aggregate or thrombus, in response to bleeding. The pathological extension of hemostasis – thrombosis – can have detrimental consequences, especially when resulting in blockage of a blood vessel, causing an ischemic stroke or myocardial infarction. Numerous studies demonstrate platelets play roles not only in hemostasis and wound healing, but also angiogenesis, inflammation, and cancer metastasis.[3-5]

Although platelets are considered cell fragments, due to their lack of a nucleus, they have many unique components that allow them to fulfill their multiple functions. Platelets contain several granules, which are classified as either alpha granules, dense granules or lysosomes. The alpha granules – considered the major protein storage organelle – contain a variety of pro- and anti-angiogenic factors, growth factors, cytokines and pro-coagulation factors, whereas the dense granules contain small molecules such as ADP, ATP, various ions, polyphosphates, serotonin and nucleotides.[6] Lysosomes contain proteolytic enzymes, such as arylsulfatase and acid phosphatase, and are thought to be involved in clot remodelling.[7] The factors secreted by platelets suggest platelet secretion plays an important role in determining the microenvironment of a wound site. Although global granule secretion occurs in a controlled manner, it remains unclear whether secretion of granular contents, such as pro-angiogenic versus anti-angiogenic factors, occurs in a controlled or random manner.[8, 9] Upon platelet activation either by exposure to the extracellular matrix or soluble agonists, granules are secreted resulting in release of more than 300 different types of molecules. The exact mechanism for release is still being investigated,

although it has been determined that the SNARE family of proteins are involved in granule secretion, particularly vesicle-SNARES (VAMPs)-2,-3,-7 and -8.[10-12] Additionally, the open canalicular system in platelets is a complex system of internal membrane invaginations, that provide extra membrane, which is involved in shape change and spreading of pseudopodia, and has been reported to contribute as part of the mechanism for granular secretion.[13]

The dense tubular system (DTS) is one of the major intracellular calcium stores in platelets, similar to the endoplasmic reticulum (ER) in other cell types. Calcium influx within platelets is not fully understood, but has been grouped into 3 categories, based on the stimulus for the calcium channel: receptor operated calcium channels (ROC), second messenger operated calcium (SMOC) channels and store operated calcium channels (SOC). ROC are activated by agonists, inducing a conformational change in the channel resulting in extracellular Ca^{2+} influx, and include several ion channels, further discussed in section 1.2.4.[14-16] SMOC are activated by second messengers such as diacylglycerol (DAG), resulting in extracellular Ca^{2+} entry.[17-19] SOC are activated when calcium stores, such as the DTS, are depleted as a result of activation by inositol triphosphate (IP_3), which occurs as a result of agonist stimulation; this category includes the Orai1 channel and the Ca^{2+} sensor: stromal interaction molecule 1 (STIM1) which are thought to create a complex that refills the DTS after Ca^{2+} depletion.[18, 20, 21] Calreticulin is thought to be the protein responsible for sequestering calcium within the DTS, and is regulated by several downstream messenger molecules that results from platelet activation.[22] Platelet activation will ultimately result in phospholipase C generation, which will generate IP_3, whose receptors (IP_3R type II) are found on the DTS, resulting in Ca^{2+} release. This release of Ca^{2+} results in activation of glycoprotein (GP) IIb/IIIa, reorganization of the cytoskeleton, and granule secretion, thereby playing an important role in platelet activation and aggregation.[23]

1.1.3 Platelet Life Cycle

Platelets are derived from megakaryocytes, in a complex process called thrombopoiesis. This process allows a megakaryocyte to split into several thousands of platelets, whose resulting life span is then about 7-10 days. Megakaryocytes are polyploid cells, who make up less than 0.1% of cells in the bone marrow. Thrombopoietin (TPO) is the primary regulator of thrombopoiesis, thought to act in conjunction with other factors, including IL-3, IL-6, and IL-11, which allow for megakaryocytes to maintain a constant platelet mass.[24]

Thrombopoiesis involves the complex process of cellular remodelling in order to generate proplatelets that bud off and then mature into platelets. Before starting proplatelet development, megakaryocytes will enlarge to approximately 100μm in diameter and become filled with high concentrations of ribosomes, to allow for generation of platelet specific proteins. TPO will bind to its receptor, c-Mpl thereby promoting megakaryocyte endomitosis, which causes the enlargement of the cell. The megakaryocyte will undergo significant maturation in this process, and results in the formation of the demarcation membrane system (DMS) – an interconnected network of cisternae and tubules. The dense tubular network and open canalicular system of platelets are formed before the beginning of the proplatelet formation process.[25] Some proteins, such as vWF and fibrinogen receptors are transported to the megakaryocyte surface, while others are packaged into their respective platelet-specific granules.[26, 27] This preparation and reorganization allows the megakaryocyte to begin formation of pro-platelets and thereby thrombopoiesis.

Through the cloning of TPO and its receptor, c-Mpl, *in vitro* studies have shed light on the proplatelet formation process, and even visualized the process, and thus have been confirmed to be similar to systems seen *in vivo*.[28] Just prior to proplatelet formation, the microtubules in the megakaryocyte will group into a mass just beneath the cortical plasma membrane, the microtubules

will then align into bundles initiating formation of pseudopodial extensions, and signalling the initiation of proplatelet formation.[24] Microtubules will merge into thick linear bundles that fill the shaft of the proplatelet as they elongate and the blunt pseudopodial extension thins. At the far end, loops will form from the microtubules, which re-enter the shaft, and this process will result in platelet-sized swellings, however, these will not bud off into platelets. The proplatelet will then develop bulbous platelet-sized structures at the end of the pseudopodia, which are in fact the primary sites of platelet assembly and release, in contrast to the swellings formed along the proplatelet shaft. Organelle and granule transport occurs along the shaft of the proplatelet, the travel of which occurs in a discontinuous manner. Evidence suggests that the organelles are moved directly by microtubules, but the movement is independent of actin.[29] Organelles and granules travel along microtubules and then slide bidirectionally in relation to other motile filaments to indirectly move along proplatelets. In mice, after complete conversion of megakaryocyte into a proplatelet network, a retraction occurs, resulting in release of individual proplatelets from the mass.[24] They are released as chains of platelet-sized particles, connected by linear microtubule bundles.

At the end of their 7-10 day life span, platelets are cleared from the body by the liver, spleen or immune system. The change in platelet glycan composition is thought to be responsible for their survival *in vivo*, particularly the loss of sialic acid – desialylation –typically of GPIbα is thought to be the trigger event for clearance by the Ashwell-Morell receptor (AMR). The AMR is a transmembrane heterooligomeric glycoprotein complex, whose expression is limited to hepatocytes and is located on the vascular side of the hepatocyte cell surface. It was originally thought to be involved in clearance of glycolipids and glycoproteins, until studies with AMR knockout mice demonstrated no increase in glycolipid or glycoprotein buildup, leaving the

function of the AMR unclear.[30] From studies on refrigerated – or cold-stored – platelets, demonstrating an increased function but decrease in platelet survivability, it is now known that the AMR clears platelets with reduced levels of α2,3-linked sialic acid.[31] In addition to clearing platelets, the uptake of desialylated platelets by the AMR activates the induction of TPO mRNA transcription by hepatocytes. In their study, Grozovsky et al., were able to demonstrate using a combination of knockout mice and antibodies, that the uptake of desialylated platelets stimulates TPO mRNA expression via the JAK2-STAT3 signalling pathway.[32] Hepatocytes are a major source of TPO, representing an important feedback mechanism regulating platelet production.[32]

1.1.4 Platelet Activation and Aggregation

Platelets are primarily responsible for maintaining hemostasis, which upon vascular injury and the exposure of subendothelial matrix is initiated by a quick, 1-2 minute, response in formation of the platelet plug, or aggregate. This requires a coordinated series of steps divided into: 1. binding to the injured site (initiation phase), 2. recruitment and activation of nearby platelets (extension phase), and 3. stabilization of the aggregate, to allow for prevention of bleeding.

Exposure of extracellular matrix (ECM) components, such as von Willebrand factor (vWF), collagen, fibronectin, thrombospondin and laminin, consequent to vessel damage are the initiators of platelet adhesion. Platelet membrane receptors are responsible for the initial binding to the vessel wall, subsequently forming a layer which initiates thrombin generation and subsequent aggregate formation.[33] Initial binding of platelets is mediated by the platelet surface receptors: GPIb/IX/V complex and collagen receptors, GPVI and GPIa/IIa (integrin $\alpha 2\beta 1$), and by the exposed vWF and fibrillar collagen at the vascular site. These factors highly depend on the rate of flow experienced, at low shear rates, adhesion primarily involves fibrillar collagen, fibronectin and laminin, but at higher shear rates, adhesion is primarily mediated by vWF interaction with the GPIb/IX/V complex.[34] This complex is comprised of 4 subunits, GPIbα and β, GPIX and GPV. GPIbα is essential for vWF binding, while GPIbβ and GPIX aide in assembly and anchoring of the complex. The interaction with vWF decelerates platelets, allowing the collagen receptors to bind to collagen, thereby firmly adhering to the surface. GPIa/IIa and GPVI are the two main collagen receptors in platelets, with both receptors being required to activate platelets.[35] The function of the GPIa/IIa receptor, is primarily to induce a strong attachment of the platelet to collagen. This relationship is so strong, that inhibiting GPIa/IIa has been shown to inhibit collagen induced aggregation.[36] GPVI acts more as a signalling molecule that is

responsible for activation of integrins, granule secretion and thromboxane A_2 (TXA_2) formation which fully activates platelets.[37] Inhibition of GPVI has been demonstrated to inhibit thrombus formation on collagen coated surfaces.[38]

The next phase, platelet activation, begins with the binding of the receptors on the platelets to the exposed subendothelial matrix, resulting in shape change of the platelet, from their resting discoid shape to the spherical shape that will then extend pseudopodia. Again, the collagen and vWF receptors play an important role in this step to ultimately mediate thrombus formation. The collagen receptors GPIa/IIa and GPVI, working together with the GPIb/IX/V complex help initiate platelet activation, via an intracellular signalling process referred to as "inside-out" signalling.[35] Inside-out signalling results in activation of GPIIb/IIIa (integrin α_{IIb}/β_3), which is activated by the binding of vWF to the GPIb/IX/X complex. GPIIb/IIIa is the most abundant receptor on the platelet cell surface with 40,000-80,000 copies, with additional pools of the receptor stored within the alpha granules and the open canalicular system.[39] The activation of GPIIb/IIIa induces a conformational change in its structure, resulting in a high affinity binding site for circulating soluble fibrinogen, allowing the formation of stable bridges between platelets and thus aggregate formation, making this the final common step leading to platelet aggregation.

After the platelet monolayer covers the exposed vWF and collagen, the recruitment of nearby platelets and aggregate formation begins. The recruitment of nearby platelets is made possible by granule secretion and subsequent accumulation of soluble agonists including ADP, TXA_2, matrix metalloproteinase-2 (MMP-2) and thrombin.[40-43] These mediators act via G-protein coupled receptors (GPCRs), which are central to the second phase of platelet-dependent thrombus formation. G-protein mediated signalling, activated by the mediators can further increase their own formation and release, acting as a feed-forward mechanism amplifying the initial signals.

This results in the rapid activation and recruitment of platelets into a growing thrombus. Activation of certain GPCRs stimulates phospholipase C (PLC) activation resulting in increased $[Ca^{2+}]_i$, as a result of PLC's hydrolysis of phosphatidylinositol 4,5 bisphosphate (PIP_2) into DAG and inositol triphosphate IP_3 signalling.[44] The increase in intracellular calcium in turn activates protein kinase C (PKC), which plays a role in cytoskeletal reorganization and thus the platelet shape change, as well as phosphorylation of downstream proteins involved in granule secretion and GPIIb/IIIa activation.[33, 45]

Stabilization phase is the last phase in aggregate/thrombus formation and refers to the signalling events promoted by close contact of the recruited platelets to withstand the high-shear environments of an artery by further increasing the number of contacts between aggregate-trapped platelets.[46] This stage is initiated by the conformational change of GPIIb/IIIa which induces an intracellular signalling process known as "outside-in" signalling. The glycoproteins and the GPCRs have the ability to crosstalk, and as a result GPCRs activate integrins and regulate the integrin outside-in signalling via $G\alpha_{13}$ subunit of GPCRs. This subunit interacts with the β3 cytoplasmic domain of the GPIIb/IIIa, making it necessary for outside-in signalling. The GPCR/$G\alpha_{13}$ and integrin outside-in signalling pathway are coordinated to regulate RhoA, a member of the Ras family, critical in platelet shape change, granule secretion and platelet spreading.[45, 47]

1.2 Coagulation

As part of vessel injury, platelets are not only involved in primary hemostasis – the act of formation of a platelet plug to arrest bleeding – but also secondary hemostasis, also referred to as the coagulation cascade. There are two pathways of the coagulation cascade, the extrinsic pathway (or tissue factor pathway) and the intrinsic pathway (or contact activation pathway), both of which ultimately lead to fibrin formation. Coagulation factors are typically denoted by Roman numerals, with the use of a lowercase "a" indicating the active form. The process of coagulation can be divided into three general phases: 1. responding of coagulation factors to form fibrin strands 2. the conversion of prothrombin to thrombin and 3. conversion of fibrinogen to fibrin, which stabilizes the platelet plug formed during the hemostatic response.

Despite the presence of the intrinsic pathway, it may not be a necessary part of coagulation *in vivo*, such that absence of factor XII in patients do not have any hemorrhagic disorder, although this may serve as a compensatory mechanism in these individuals.[48] The contact pathway instead acts as a powerful tool for studying coagulation *in vitro*. The intrinsic pathway is involved in sustaining coagulation via Factors XI, VIII and V as a cofactor of the prothrombinase complex. Serine protease factor IXa cleaves factor X into its active form Xa. Activation of factor X require a phosphatidyl serine (PS)-containing membrane surface, to allow for assembly with the factors VIIIa and IXa.

As platelets accumulate at the site of injury due to exposed collagen, the exposed tissue factor from the subendothelial matrix is responsible for thrombin generation. Activated platelets, and endothelial cells, can secrete the enzyme necessary for conversion of inactive tissue factor into its active form; in certain cases of direct tissue damage, tissue factor may already exist in its active form.[43] Tissue factor will become bound by circulating activated factor VII (fVIIa) and form a

complex. The complex will then activate factor IX to IXa, which will bind to factor VIII. Factor VIIIa will activate factor X to form factor Xa but can also be generated by the factor VIIa complex with tissue factor. Factor Xa will then bind factor V on membrane surfaces, allowing conversion of factor II (prothrombin) to factor IIa (thrombin). Thrombin and FXIII will then cleave soluble factor I (fibrinogen) to insoluble fibrin, stabilizing the clot.

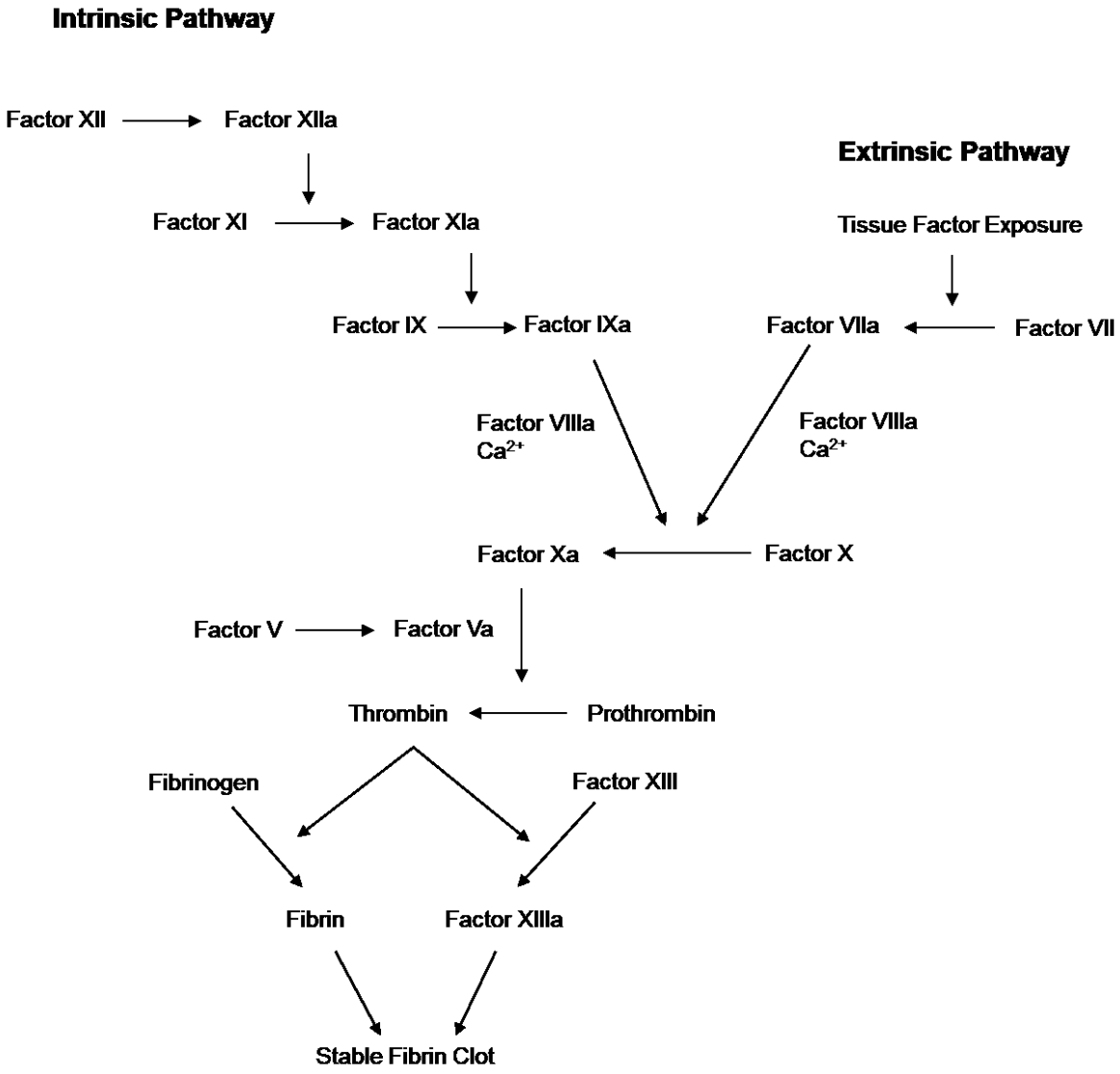

Figure 1. Coagulation cascade.

1.3 Negative Regulation of Platelet Function

1.3.1 The Role of the Healthy Endothelium

The endothelium is the inner most, single cell layer that makes up every blood vessel. The endothelium, made up of endothelial cells, is crucial to maintaining vascular health, alongside regulating blood pressure, and preventing atherosclerosis. The endothelium secretes many factors, not only involved in regulation of blood flow, but also factors that effect platelets. Endothelial cells play a key role in maintaining a balance between activation and inhibition of hemostasis. The healthy endothelium prevents platelet attachment and inhibits proteins involved in initiation of coagulation and platelet aggregation. The major factors responsible for this inhibition are prostacyclin (PGI_2) and nitric oxide (NO), the two most potent endogenous inhibitors of platelet adhesion and aggregation. Additionally, evidence suggests that prostacyclin and nitric oxide synergize to inhibit platelet aggregation.[49] PGI_2 and NO stimulate adenylyl cyclase and guanylyl cyclase activity, respectively, resulting in intracellular increases of cAMP and cGMP, respectively.[49-51] Down-stream of this, the signalling involves cAMP-dependent protein kinases (PKA) and cGMP-dependent protein kinases (PKC).[52] Physiologically, platelets contain high levels of PKA and PKG, selective stimulation of which correlates with platelet inhibition. PKA and PKG mediated signalling involves: inhibition of GPIIb/IIIa activation and fibrinogen binding, phosphorylation of VASP and IRAG, decreased actin polymerization and myosin light chain kinase activity. Additional evidence demonstrates involvement in decreasing $[Ca^{2+}]_i$ via inhibition of intracellular calcium channels.[53] There is also evidence demonstrating phosphorylation of Ser166 of the GPIb/IX/V complex in platelets by a cAMP-dependent kinase activated by PGI_2, inhibiting platelet action.[54]

Another manner by which endothelial cells regulate platelet activity is by expression of CD39. CD39 is an ADPase responsible for degradation of ADP released by erythrocytes and aggregating platelets, thereby contributing to regulation of hemostasis under physiological conditions.[33, 55] In inflammation, the regulation by PGI_2 and NO is impaired due to cytokine-inducible adhesion molecules becoming expressed on the surface of affected endothelial cells. This allows bypassing of the system normally in place and binding and activation of platelets. Additionally, these activated endothelial cells can release vWF and P-selectin, which are both ligands for GPIbα.[35]

1.3.2 Endothelial Nitric Oxide Synthase

Endothelium-derived relaxing factor (EDRF), a factor that was found to induce relaxation of blood vessels in response to acetylcholine, was independently identified as NO by two groups, Moncada and Ignarro.[56-58] Today the importance of endothelial-derived NO is well established, contributing to the vasodilatory, antithrombotic and atheroprotective properties of the endothelium.

NO is a soluble gas with a half life of 7 seconds that diffuses freely across membranes.[59] NO is generated by the family of nitric oxide synthases (NOS), of which there are three isoforms of NOS, neuronal (nNOS or NOSI), inducible (iNOS or NOSII) and endothelial (eNOS or NOSIII). Under physiological conditions the main source of NO is generated by eNOS in endothelial cells.[60] eNOS can be localized within lipid rafts in the plasma membrane of the endothelial cell, called caveolae. The activity of eNOS is inhibited by interaction with caveolin-1, a caveolae coat protein. Dissociation from caveolin-1 is caused by rising $[Ca^{2+}]_i$, which then allows interaction of eNOS with calcium-activated calmodulin, thus demonstrating calcium-dependent activation.[61] eNOS activity can also be moderated by calcium-independent mechanisms via shear stress-induced phosphorylation or by other proteins such as VEGF or heat shock protein 90.[62]

eNOS (and other NOS isoforms) utilize L-arginine as its substrate, alongside molecular oxygen and reduced nicotinamide-adenine-dinucleotide phosphate (NADPH) as co-substrates. Cofactors of the enzyme are flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6R)-5,6,7,8-tetrahydro-L-biopterin (BH_4). eNOS transfers the electrons from NADPH via FAD and FMN in the C-terminal reductase domain to the heme in the amino-terminal oxygenase domain, where BH_4, oxygen and L-arginine are bound.[63, 64] At the heme, the electrons reduce

and activate oxygen and oxidize L-arginine to L-citrulline and NO. This occurs in a two-step process, where the first step is hydroxylation of L-arginine to N^{ω}-hydroxy-L-arginine, and the second step oxidized N^{ω}-hydroxy-L-arginine to NO and L-citrulline.[62, 65, 66] Soluble guanylate cyclase and the generation of cGMP play a major role in the physiological signalling pathway activated by NO.[67, 68]

Endothelium-derived NO plays a critical regulatory role in the maintenance of cardiovascular homeostasis. NO is able to inhibit both platelet aggregation, and platelet adhesion to the endothelium. Besides this, endothelial-generated NO is critical for adaptive vascular remodelling to changes in flow, inhibiting leukocyte adhesion and vascular inflammation, as well as inhibiting vascular smooth muscle cell proliferation.[69-71] However, under pathophysiological conditions, a process referred to as eNOS uncoupling can occur. This uncoupling can occur as a result of BH_4 oxidation, depletion of L-arginine, and accumulation of endogenous methylarginines.[62, 72] eNOS uncoupling can occur alongside endothelial dysfunction, resulting in a decreased ability to generate adequate amounts of NO while simultaneously generating reactive oxygen species. This ultimately results in NO reacting with $O_2^{-\cdot}$ (superoxide) to generate $ONOO^-$, peroxynitrite. Oxidative stress has also been shown to convert eNOS from NO-producing enzyme to $O_2^{-\cdot}$-producer.[62]

1.3.3 Nitric Oxide Generation in Platelets

In 1990, platelets were determined to be capable of generating NO after a series of pharmacological studies using L-arginine.[73, 74] These studies demonstrated that L-arginine, the substrate for NOS, was able to inhibit platelet aggregation induced by agonists such as collagen, ADP and arachidonic acid. Co-incubation with L-arginine also induced cGMP production, and NO formation was measured using spectrophotometric analysis, all pointing to the presence of a NOS pathway in platelets. As well they were able to demonstrate, that the use of NOS inhibitors reversed these effects. Several years later, enzyme activity was measured using a porphyrinic microsensor, demonstrating the generation of NO in a concentration dependent manner when aggregated with collagen but not thrombin.[75] Additionally, they demonstrated an increase of NO production with L-arginine treatment, and blockage of the NO signal when treating with N^G-monomethyl-L-arginine, a NOS inhibitor.

Platelet NO-generation has been primarily attributed to the eNOS isoform of NOS.[73, 76] NO is one of the major endogenous inhibitors of platelet aggregation, and since platelets are able to generate NO it suggests their ability to negatively-feedback their own aggregation and limit their function in an autocrine manner. More recent evidence has demonstrated the presence of eNOS within platelets using anti-eNOS antibodies, suggesting a model of platelet subpopulations. These subpopulations are considered to be platelets that are NO-producers and non-NO-producers.[77] This study provides evidence of NO-generation and the presence of eNOS in platelets, alongside the presence of the soluble guanylate cyclase pathway. Additionally, the evidence demonstrates that NO generation increases upon activation of platelets, and acts to limit thrombus formation, thus confirming their autocrine negative-feedback ability.

1.4 Ion Channels in Platelets

Ions channels are known for the well-established role of maintaining and regulating ionic permeability. Ionic permeability is what allows for control of membrane potential, cell volume, and control of intracellular ion concentration, especially the second messenger, $[Ca^{2+}]_i$. Much like other factors in platelets, the functions of ion channels may influence hemostasis and thrombosis. Due to the nature and size of the platelet, this makes studying ion channels via patch-clamping a challenge, and instead megakaryocytes or megakaryocytic cell lines are often used as platelet surrogates.

1.4.1 P2X1

The P2X family of ion channels, are non-selective cation channels, that are thought to be exclusively activated by the binding of ATP, under physiological conditions.[78] Of the P2X family, only P2X1 has been identified in platelets, as well as megakaryocytes.[79] These channels are activated by ATP, resulting in Ca^{2+} and Na^{+} influx, and due to the influx of extracellular Ca^{2+}, represent the fastest mechanism where tissue damage leads to an increase in $[Ca^{2+}]_i$ in the platelet. ATP is among several factors released by cells following an injury to their membrane, such as what would occur from vascular injury, thereby stimulating P2X1 and the pro-aggregatory response of platelets.[80] ATP is also among the factors that are secreted from platelet dense granules, thereby contributing to feeding-forward platelet aggregation. *In vitro,* the influx of Ca^{2+} due to P2X1 activation has been demonstrated to result in shape change, granule secretion and cause minor levels of aggregation.[81-83] The direct and amplifying effects of P2X1 are thought to be due to Ca^{2+} influx, but not linked to the Na^{+} influx, or subsequent depolarization caused. P2X1 activity is resistant to classical platelet inhibitors such as PGI_2 and NO, thereby conferring its importance in early stage $[Ca^{2+}]_i$ elevation.

1.4.2 Ionotropic Glutamate Receptors

In addition to the ATP ligand-gated ion channel, several other ligand-gated cation channels have been described in platelets. Platelets secrete several neurotransmitters from dense granules, including serotonin, glutamate and acetylcholine. There is evidence for the presence of all three types of ionotropic glutamate receptors, NMDA, AMPA and kainate, named after their respective ligands, in platelets.[84] Based on *in vitro* studies, evidence suggests that glutamate alone does not stimulate any functional platelet responses, but glutamate, kainate or AMPA have been shown to enhance responses to agonists such as TXA_2 and thrombin receptor-activating peptide (TRAP).[14, 85-87] Additionally, in studies using CNQX, an AMPA antagonist, activation of platelets by TRAP was inhibited. AMPA and kainate channels are closely related by their subunits and both channels are Na^+ permeable but Ca^{2+} impermeable. AMPA and kainate have been demonstrated to potentiate platelet activation, but due to the absence of Ca^{2+} involvement, their mechanism of action remains unclear.[14, 85] NMDA channels, in contrast to AMPA and kainate mediate the influx of Ca^{2+} and Na^+.[85] Despite permeability to Ca^{2+} and Na^+, at resting potential NMDA channels are typically blocked by Mg^{2+} ions, thus requiring depolarization to generate Ca^{2+} influx, although the overall increase is thought to be small.[88] Evidence from studies in megakaryocytes has demonstrated that MK-801, NMDA receptor antagonist is able to bind to megakaryocytes, inhibit human megakaryocyte development, and inhibit proplatelet formation in the Meg-01 cell line.[89, 90] NMDA itself presents mixed evidence of either having no effect on platelet responses, or inhibiting platelet aggregation.[85, 88, 91]

1.4.3 Organellar Ion Channels

Organellar ion channels that are present in platelets include: IP_3 receptors and the two-pore channel (TPC).[14] Type II IP_3 receptors sit on the intracellular calcium store of platelets, the dense tubular system, and upon activation result in the release of Ca^{2+} from the DTS. IP_3 receptors are thought to be non-selective cation channels which are co-activated by cytosolic IP_3 and Ca^{2+}.[14] From studies on whole-cell patch clamping of megakaryocytes, current evidence suggest that IP_3 release into the cytoplasm generates a sustained and oscillatory inward cation current, although the cause and identity of this conductance remains unknown.[92, 93] IP_3 receptor signalling can be completely inhibited by PKA or PKG, which are the major mechanisms of PGI_2 or NO inhibition of platelets, thereby inferring inhibition of platelet function.[94, 95] In platelets, the TPC is another Ca^{2+} permeable channel, activated by nicotinic acid adenine dinucleotide phosphate (NAADP). Not much is known about this channel and most of the functional information comes from studies in mice. One of such studies demonstrated that the deletion of CD38, an ADP ribosyl cyclase that is responsible for generation of NAADP, inhibits Ca^{2+}-signalling, and PS exposure with decreased thrombus stability in response to thrombin.[14, 96]

1.4.4 Gap Junctions

In various cell types, cell-to-cell communication is made possible by gap junctions in situations of close contact. This holds true for platelets, who have been demonstrated as having gap junctions allowing for cell-to-cell communication during thrombus formation.[97] Previous studies have demonstrated the diffusion of calcein between platelets in a thrombus, using fluorescence measurements.[98] Others have demonstrated the presence of gap junction like structures between membranes of aggregated platelets via electron microscopy.[98] In studies using connexin blockers, which block gap junctions, the results demonstrate in a decrease in

platelet response, suggesting a role of gap junctions in thrombus formation.[99] Other work stands in direct contrast with this data, and demonstrates reduced bleeding, increased aggregation and enhanced thrombosis, with the use of connexin blockers, instead suggesting a role for gap junctions in inhibition of the platelet response.[98]

1.4.5 Voltage Gated Potassium Channels

Platelets, like most cells, contain depolarization-gated K^+-selective (K_V) channels. In platelets, a single alpha subunit $K_V1.3$, is responsible for formation of the channel.[14, 100] The channels display a threshold for activation around -60mV, and are steeply voltage-dependent in ranges of -40mV – -10mV. The conductance sets the membrane potential of platelet at approximately -50mV, but are also thought to contribute to membrane potential during agonist stimulation, which upon blockade has demonstrated a reduction in Ca^{2+} entry.[101, 102] Additionally, other evidence proposes K_V channel involvement in lymphocyte adhesion through direct interaction with β_1 integrins.[14, 103, 104]

1.4.6 Transient Receptor Potential Channels

Transient Receptor Potential Channels, or TRP channels involve approximately 30 ion channels in their family, displaying various permeabilities and thus playing diverse roles. Human platelets are thought to have: canonical TRP (TRPC)1, 3, 4, 5 and 6, and TRPV1. Of interest is the TRPC family, who upon activation result in Na^+ and $Ca2^+$ entry, and are thought to interact with Ca^{2+}-handling proteins such as the IP_3R type II.[105] TRPC6 can colocalize with TRPC1 and is thought to have a role in platelet SOCE, by being part of the complex involving STIM1, Orai1 and Orai2. TRPC6 is also thought to be involved in SMOCE where it acts together with TRPC3, where activation of TRPC6 may induce DAG, a decrease in PIP_2 and possibly protons.[105-108] However, studies in mice deficient in TRPC6 offer conflicting evidence, with one study suggesting

no changes in platelet Ca^{2+} nor functional responses.[109] Another study reported the finding that TRPC6 deletion in mice, resulted in increases in bleeding time and a small decrease in thrombosis.[106, 110] TRPC6 has otherwise been suggested as a candidate that is activated at higher concentrations of thrombin to allow cation entry, thus possibly explaining differences in results between groups.[14]

1.4.7 Chloride Channels

Previous studies using patch clamping on platelets and megakaryocytes have demonstrated the presence and functional expression of chloride channels in the plasma membrane that are activated by Ca^{2+} and depolarization.[111, 112] However, it remains unclear whether or how chloride channels cause an increase in $[Ca^{2+}]_i$. The chloride channels are thought to be involved in regulation of volume and resting membrane potential in platelets.[101, 113] Due to a lack of specific chloride channel blockers, a more recent study has chosen to use extracellular Cl^- ion substitution to study its effects.[114] Evidence from this study demonstrates that the reduction of extracellular Cl^- will decrease rate of platelet activation and aggregation, and decrease in platelet agonist mediated $[Ca^{2+}]_i$.[114]

1.4.8 Other Channels

Evidence suggests the presence of calcium-activated potassium (K_{Ca}) channels platelets, based on two separate studies, one using a fluorescent indicator, and the other using electrophysiological methods.[87, 115] This will be further discussed in section 1.5.

1.5 K_{Ca} Channels

1.5.1 History, Discovery and Subtypes

In 1958, in an attempt to explain why the inhibition of glycolysis in red blood cells caused a rapid increase in K^+ efflux, that could not be explained by the velocity of regular physiological exchange, Gardos made the first finding of calcium-activated potassium (K_{Ca}) channels.[116] He found that erythrocytes exposed to EDTA could not undergo potassium efflux in the absence of calcium, and shortly thereafter confirmed these results with the use of other calcium chelators.[116, 117] These findings constitute the first description of the family of channels referred to as calcium-activated potassium channels, and for historical significance is occasionally referred to as the Gardos channel, especially in erythrocytes. Today, it is known that there are 3 subtypes of calcium-activated potassium channels – large conductance (BK_{Ca}), intermediate conductance (IK_{Ca}) and small conductance (SK_{Ca}) – based on their relative sizes of conductance.

SK_{Ca} channels have conductance of 10-40pS and were first identified in the brain. There are 4 members of this family of channel, all encoded by *KCNN1-4* genes, with *KCNN4* being considered distinct due to size of conductance, and therefore categorized as IK_{Ca} channels, also referred to as SK4. SK_{Ca} channels are comprised of four subunits, each with 6 transmembrane domains (S1-6), the pore forming region (S5-6) is highly conserved among all K_{Ca} channel family members. SK_{Ca}, including IK_{Ca}, channels are voltage insensitive, thus independent of changes in membrane potential. The Ca^{2+}-sensitivity of these channels comes from being bound to calmodulin in the C-terminal domain of every subunit, conferring a sub-micromolar sensitivity to Ca^{2+}, in the 250-900nM range. Expression of SK_{Ca} channels, including IK_{Ca}, has been demonstrated in neurons, endothelium, epithelium and a variety of smooth muscle cells, excluding the vascular smooth muscle (VSM) cells. The Gardos channel in erythrocytes is classified as the IK_{Ca} channel

subtype, based on its conductance of about 39pS.[118] The coupled calmodulin senses changes in the intracellular calcium concentration ($[Ca^{2+}]_i$), with induction of a conformational change which involve the interlacing of cytoplasmic loops leading opening of the pore.

BK_{Ca} channels are distinct from the IK_{Ca} and SK_{Ca} channels, because they are sensitive to changes in voltage as well as changes in calcium concentration. They are composed of four alpha and four beta subunits, with the alpha units, composed of 11 hydrophobic domains (S0-S10) with formation of the pore between subunits S5 and 6.[119, 120] The membrane spanning portions are made up of S0-S6, with an extracellular N-terminus, with the S4 domain likely presenting the voltage sensor due to the presence of several positively charged amino acids. S7-10 are thought to be in the cytoplasm and form the C-terminal tail of the BK_{Ca} channel. The S5-6 linker is thought to make up the pore-forming region, and is made up of 2 alpha-subunits, that may form a functional channel even in the absence of beta-subunits.[121] Other studies have suggested that the alpha-subunit has an intrinsic sensitivity to $[Ca^{2+}]_i$, possibly conferring a "calcium-bowl" in the tail region, although the channel's response to calcium has been demonstrated even in the absence of the tail, indicating that the "calcium-bowl" is not the exclusive calcium sensor.[122-125]

The activation of the BK_{Ca} channel by membrane potential still depends on $[Ca^{2+}]_i$, if calcium is low the channel responds exclusively to changes in voltage, behaving like a K_V channel.[126, 127] The voltage sensing mechanism is independent of calcium binding, but will shift many of the voltage dependent parameters to a more negative range, in higher concentrations of calcium, allowing the channel to function at physiological ranges of membrane potential.[128]

BK_{Ca} channels are regulated by phosphorylation via a variety of kinases, including PKA, PKG-I and PKC.[129, 130] Phosphorylation serves to modulate the channels calcium and voltage sensitivity. PKA and PKG-I most often increase the probability of the open state of the channel,

these kinases may also indirectly activate the channel by phosphorylation of phospholamban.[131] Alternatively, PKC phosphorylation inhibits the BK_{Ca} channel in smooth muscle.[132]

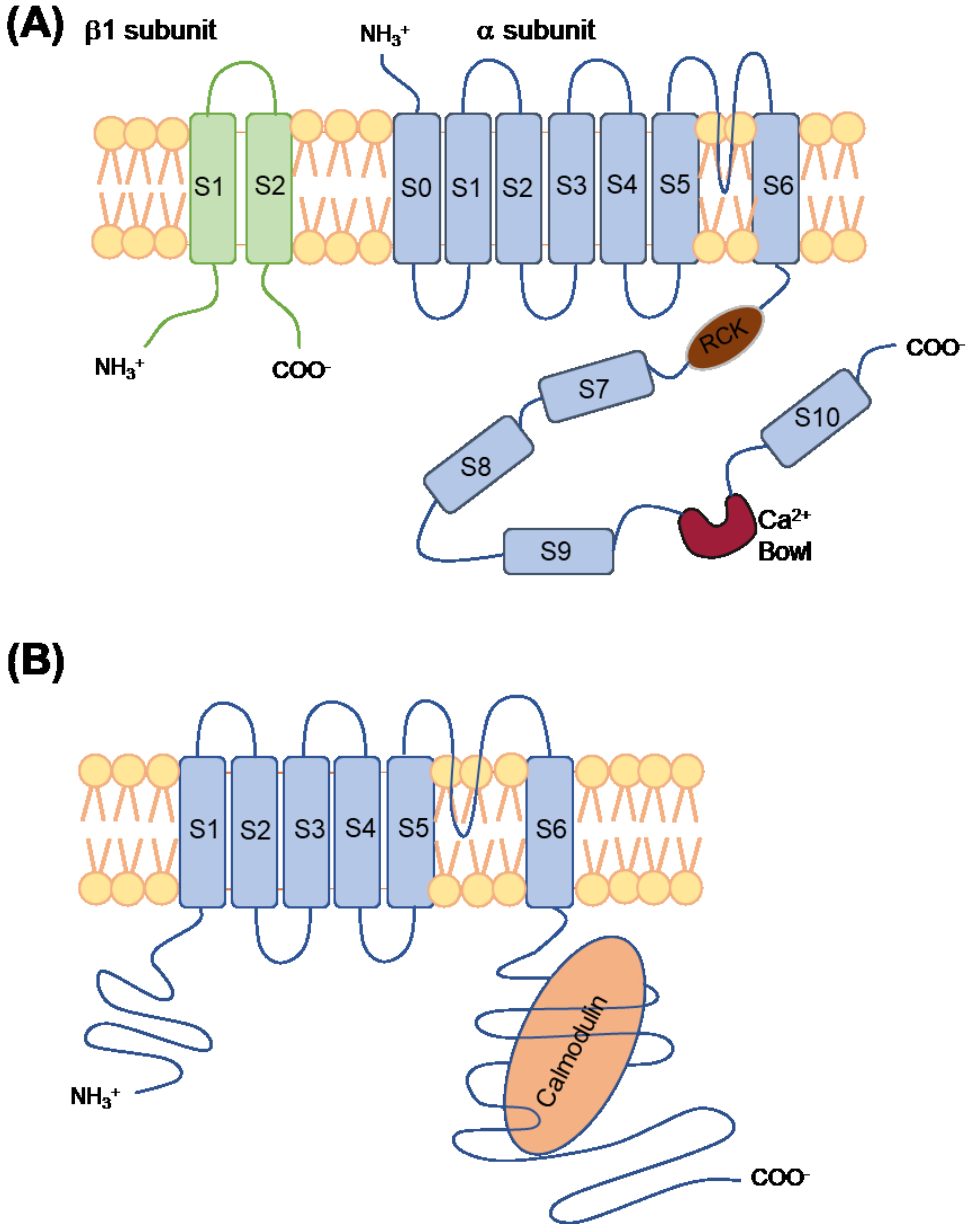

Figure 2. K_{Ca} Channel Structures. (A) BK_{Ca} channel structure, as imbedded in the membrane with 2 transmembrane domains in the β1-subunit and transmembrane components of the α-subunit S0-6, with the pore in between S5 and 6. (B) SK_{Ca} channel structure, consisting of 6 transmembrane domains, with the pore region forming between S5 and S6, and calmodulin interacting at the intracellular C-terminus. Adapted from Ledoux et al.[133]

	SK_{Ca} (SK1-3)	**IK_{Ca} (SK4)**	**BK_{Ca}**
Conductance	5-10 pS	20-40 pS	150 pS
Genes	*KCNN1-3*	*KCNN4*	*Slo* gene-encoding α-subunit
Calcium sensitive?	Yes	Yes, via calmodulin	Yes, via calmodulin
Respond to voltage	No	No	Yes
Activators	Riluzole 1-EBIO NS309 CyPPA	Riluzole 1-EBIO NS309 SKA-31	NS1619 NS1608 Estrogen
Blockers	Apamin UCL 1684 Bicuculline	TRAM-34 Charybdotoxin Clotrimazole	Iberiotoxin Charybdotoxin Ethanol
Expression	Endothelium Neurons Nonvascular smooth muscle Epithelium	Endothelium Epithelium Lymphocytes Erythrocytes	Vascular and non-vascular smooth muscle Adrenal gland

Table 1. Summary of K_{Ca} channel expression.

1.5.2 K_{Ca} Mechanism of Action

Studies in endothelial cells have determined the distinct presence of IK_{Ca} and SK_{Ca} channels, with the clear absence of BK_{Ca} channels. The K_{Ca} channels present in endothelial cells are thought to contribute to the endothelial-dependent hyperpolarizing factor (EDHF), an amalgamation of mechanisms and compounds that play a role in maintaining vasomotor tone. The proposed mechanism of action of K_{Ca} channel activation, thus stems from research done in endothelial cells.

Under physiological conditions, an agonist such as, but not limited to, acetylcholine will bind to its M3 receptors resulting in stimulation of IP_3 signalling. IP_3 will stimulate Ca^{2+} release from intracellular stores, which will stimulate eNOS to generate NO, which through several downstream mediators results in inhibition of VSM contraction. The Ca^{2+} released from intracellular stores will not only stimulate NO generation but will also result in the opening of IK_{Ca} and SK_{Ca} channels.[134] The opening of these channels causes K^+ efflux, hyperpolarizing the membrane. The hyperpolarization of the endothelial cell membrane provides the driving force for non-specific Ca^{2+} entry via TRPC channels, which then feeds-forward to stimulate eNOS to generate additional NO, further relaxing the VSM (Figure 3).[109, 135-137]

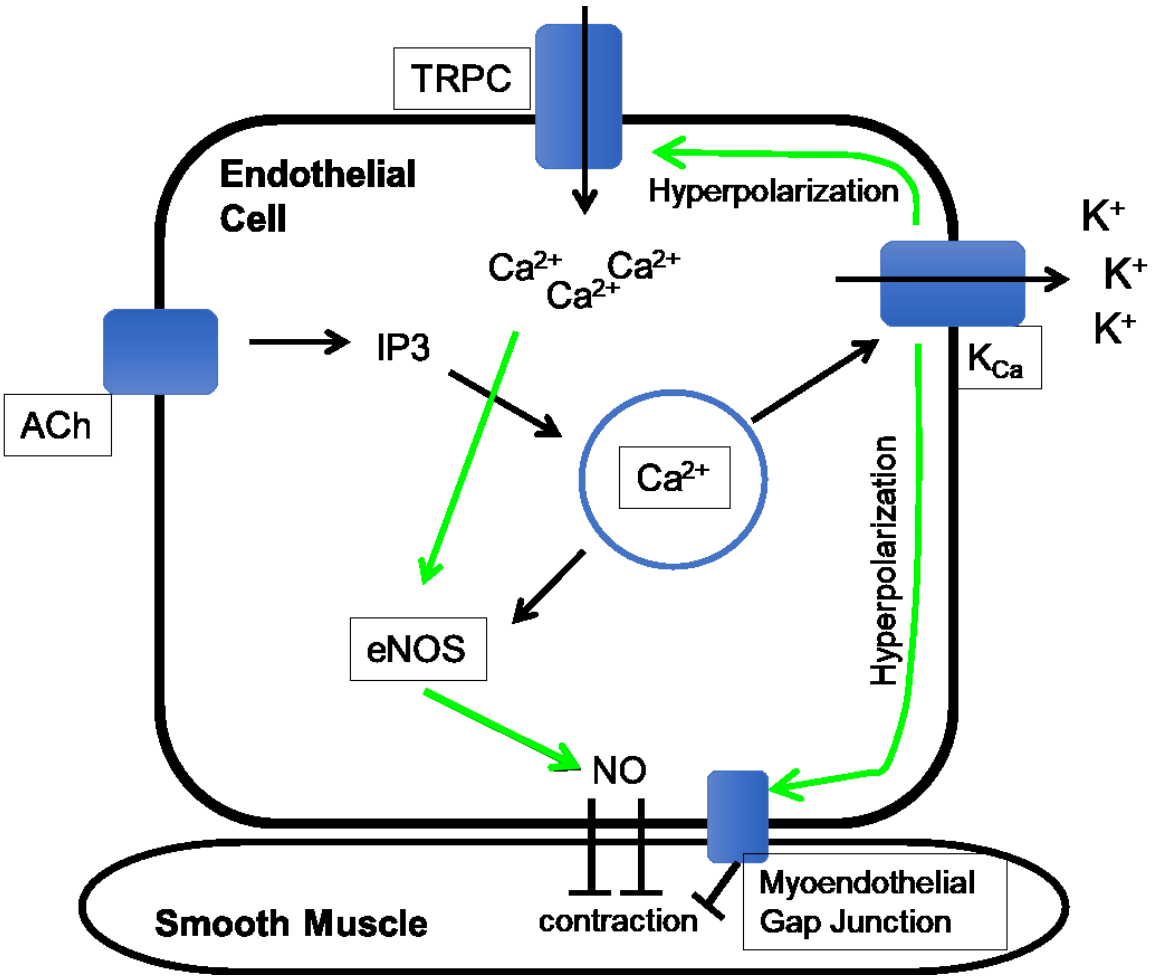

Figure 3. Mechanism of Action of IK_{Ca} and SK_{Ca} Channels in Endothelial Cells.

1.5.3 Pharmacological Modulators of K_{Ca} Channels

One of the oldest activators of K_{Ca} channels is thought to be 1-EBIO, which is capable of activating all channel members belonging to the *KCNN1-4* genetic family, this includes the SK_{Ca} subtypes as well as IK_{Ca} channels.[138] Despite being able to activate all 4 subtypes, it is more sensitive to IK_{Ca} channels, with EC_{50} values reported around 30μM, and EC_{50} values for SK_{Ca} channels reported at 5-10 times higher.[139] Current activators of K_{Ca} channels are based off the drug riluzole, which was demonstrated to activate K_{Ca} channels amongst exerting actions on many other ion channels.[138] This led to development of compounds such as NS309 and SKA-31, potent IK_{Ca} channel activators, both of whom retain the benzimidazole/benzothiazole group, suggesting that this group plays a role in conferring sensitivity to IK_{Ca} channels.[140] Two newer analogs derived from SKA-31 include SKA-121 and SKA-111, which also retain the benzimidazole/benzothiazole group, but demonstrate even higher selectivity for IK_{Ca} channels over SK_{Ca} channels.[141] Contrastingly, CyPPA and its more potent relative NS13001 have different chemical structures, but display similar selectivity for SK_{Ca} channel subtypes.[138, 142] Both compounds activate $SK_{Ca}2.3$, but not $SK_{Ca}2.1$, with varying degrees of action on $SK_{Ca}2.2$, however further analysis is required as to why these compounds differ.[142]

All K_{Ca} channel activators function by shifting the Ca^{2+} activation curve to requiring lower Ca^{2+} levels to activate the channels.[138] Kinetics evidence demonstrates that the activators do not open the channels faster, but rather allow them to stay open longer.[143] 1-EBIO has been demonstrated, via co-crystallization to bind at the calmodulin binding domain site, similarly NS309 and CyPPA have been demonstrated to do the same.[143]

In contrast, channel blockers include compounds such as TRAM-34 which targets IK_{Ca} channels, and apamin, which targets SK_{Ca} channels. Apamin, which stems from bee venom,

blocks the SK_{Ca} channels in an allosteric fashion.[144] Other more traditional, or non-allosteric, blockers of SK_{Ca} channels include NS8593, which has been demonstrated to interact deep within the pore and not at the calmodulin binding domain, but instead interacts with serine 507 and alanine 532.[145, 146] In comparison, TRAM-34, the IK_{Ca} channel blocker interacts at similarly positioned residues within the IK_{Ca} channel, as the SK_{Ca} blockers interact within the SK_{Ca} channel pore.[138, 147]

1.5.4 Discovery of K_{Ca} Channels in Platelets

The first strong evidence of K_{Ca} channels in platelets is from Aviv's group in 1989, in a study using the cationic fluorescent probe DiSC3(5).[115] The study demonstrated measurements of hyperpolarization caused by A23187, a calcium ionophore. From the results, the conclusions drawn were that: 1. K_{Ca} channels exist in platelets, 2. they are apamin insensitive but charybdotoxin sensitive, and 3. they may be involved in regulating the resting membrane potential.[115] Since apamin is an SK_{Ca} channel blocker, these results suggest that the subtype of K_{Ca} channel within platelets is the IK_{Ca} channel, not the SK_{Ca} channel.

This initial study was followed up several years later in 1995 by Mahaut-Smith, using whole cell patch clamping on platelets, in the presence and absence of calcium and under varying membrane potentials to confirm the presence of K_{Ca} channels in platelets.[87] The conclusions of this study were that calcium-sensitive potassium channels exist within platelets, at a low copy number of roughly 5-7, with a conductance of 30pS. Based on the conductance recorded, this study also suggests the subtype of K_{Ca} channels present in platelets to be IK_{Ca} channels. Additionally, Mahaut-Smith suggests that the K_{Ca} channels are not active under resting conditions but may be involved in regulating membrane potential during Ca^{2+} signalling. Another groups has confirmed the presence of IK_{Ca} channels by mass spectrometry.[148] However, despite these studies and the identification of K_{Ca} channels within platelets, their specific subtype and function in platelets remains unknown and not wholly understood.

2. HYPOTHESIS AND OBJECTIVES

2.1 Rationale

While hemostasis is important in the regulation of blood flow, and wound healing, thrombosis – uncontrolled aggregation – can have detrimental consequences. Current therapies available attempt to target thrombosis by strongly limiting the activity of platelets, and thus also limiting the hemostatic function of platelets, thus increasing the risk of bleeding in patients. As such, these therapies are typically implemented in post-thrombotic event patients, as secondary prevention, and only used in high-risk individuals as primary prevention, due to the associated risk of bleeding. Therefore, there is always a necessity for new anti-platelet therapy targets, especially a target that would limit the development of thrombosis, but limit targeting of hemostasis, thus decreasing the risk of bleeding.

Previous studies in endothelial cells, suggest a mechanism of action whereby activation of IK_{Ca} and SK_{Ca} channels activates eNOS in a downstream fashion, generating additional NO, thereby mitigating increased arteriolar pressure.[136, 149] The inhibitory role of NO on platelet function is well understood, highlighting the importance platelet eNOS, and the ability of platelets to generate their own NO. The NO generated by platelets acts as a negative feedback mechanism, limiting their own aggregation. The identification of IK_{Ca} and possibly SK_{Ca} channels within platelets and presence of eNOS, makes it possible that this same machinery would follow the same mechanism of action as in endothelial cells. Such that, the pharmacological activation of K_{Ca} channels, would cause efflux of potassium ions, resulting in hyperpolarization of the platelet membrane, subsequently activating calcium channels causing a potential localized increase in calcium, enabling activation of eNOS to generate NO. Thus, pharmacological activation of K_{Ca} channels to enhance platelet-NO generation may be a novel anti-platelet mechanism/drug target.

Targeting the negative feedback pathway of NO could preferentially inhibit thrombosis rather than both thrombosis and hemostasis, possibly decreasing the risk of bleeding that is an issue faced with current clinically utilized anti-platelet drugs.

2.2 Hypotheses

1. Pharmacological activation of platelet IK_{Ca} and SK_{Ca} channels will inhibit platelet aggregation.
2. Pharmacological activation of platelet IK_{Ca} and SK_{Ca} channels will increase platelet NO generation.

2.3 Objectives of the Study

The objectives of this study are:

1. To identify the K_{Ca} channel subtypes present in platelets.
2. To characterize the effects of pharmacological IK_{Ca} and SK_{Ca} channel activation on platelet aggregation.
3. To determine if there are any differences in platelet adhesion and aggregation in response to pharmacological K_{Ca} channel activation under laminar flow conditions.
4. To determine if there is an increase in platelet-derived NO generation in response to pharmacological activation of platelet K_{Ca} channels
5. To investigate the effects of pharmacological K_{Ca} channel activation on platelet Ca^{2+} signalling.
6. To characterize the effects of pharmacological K_{Ca} channel activation on other aspects of platelet machinery, such as dense and alpha granule secretion.

3. MATERIALS AND METHODS

3.1 Reagents

Antibodies against the IK_{Ca} or SK_{Ca} channels (clones: M20 and M75, respectively), as well as the blocking peptides for the antibodies were obtained courtesy of Dr. Shaun Sandow (Gold Coast, Queensland, Australia). Activators of IK_{Ca} or SK_{Ca} channels, SKA-31 and CyPPA, respectively, alongside the inhibitors of IK_{Ca} and SK_{Ca} channels, TRAM-34 and apamin, respectively were obtained through Tocris (Oakville, ON, Canada). Collagen, thrombin, Chronolume™ luciferin/luciferase for aggregation studies and chemiluminescence were purchased from Chrono-Log (Havertown, PA, USA). DAF-FM diacetate, the NO binding dye, as well as Fluo-4 AM, the calcium signalling dye were obtained through ThermoFisher Scientific (Rockford, IL, USA). Any supplies, except for collagen, used in the Qsense™ Quartz Crystal Microbalance experiments to study laminar flow, were purchased from Biolin Scientific (Gothenburg, Sweden). The phalloidin-568 probe, used to stain for F-actin in the laminar flow experiments and in identifying the plasma membrane for microscopy studies was purchase from ThermoFisher Scientific (Rockford, IL, USA). The antibody for p-selectin (anti-CD62P-PE, clone AC1.2), used in flow cytometry to measure alpha granule secretion, was purchased from BD Biosciences (Mississauga, ON, Canada). The marker for platelets frequently used in flow cytometry experiments, anti-CD42b-PE antibody (clone HIP1), was also purchased from BD Biosciences. Unless otherwise indicated, other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

3.2 Platelet Isolation

Prostacyclin-washed platelets were prepared from healthy human volunteers, who were free of drugs known to affect platelet function for at least 2 weeks prior to blood draw. Blood was collected via venous puncture of the arm, and 36ml of blood was collected into a 50ml tube, with 4ml previously prepared trisodium citrate inside (12μM, 9:1 ratio), and isolated according to the protocol previously established by Radomski and Moncada.[150] Prostacyclin was then added to the blood at a concentration of 0.06μg/ml, and the tube was centrifuged in an Eppendorf 5810R centrifuge (Eppendorf, Hamburg, Germany) at 250g for 20 minutes, set with acceleration at 7 and brake set at 0, to separate out the platelet rich plasma (PRP) from erythrocytes. PRP was then collected into a separate tube, to which prostacyclin was added, at a concentration of 0.3μg/ml, and then centrifuged at 900g for 10 minutes, with the acceleration and brake set as before (7 and 0, respectively). This pellets out the platelets, leaving the supernatant as platelet poor plasma (PPP). The PPP was removed and discarded, and the platelet pellet was washed without resuspension 3 times with 1ml of Tyrode's Buffer. The platelets were then re-suspended, and counted using a hemocytometer (Assistant Sondheim, Germany) and diluted to a final concentration of $2.5x10^8$ platelets/ml in Tyrode's Buffer.

3.3 Immunofluorescence Microscopy of K_{Ca} Channels in Platelets

Isolated platelets were fixed in 4% paraformaldehyde in PBS for 20 minutes and centrifuged at 900g for 10 minutes to pellet samples. Paraformaldehyde solution was removed and discarded, and platelets resuspended in phosphate-buffered saline (PBS). Coverslips were coated with poly-L-lysine for 15 minutes, and upon drying of the coverslips, 100 µl of platelets were pipetted onto the coverslip and allowed to adhere for 30 minutes. Samples were then permeabilized using 0.1% Triton X-100 in PBS for 10 minutes, using the inverted coverslip on droplet technique (Figure 4). Coverslips were then washed with Buffer Solution (PBS+1%BSA+0.05%Tween20) 3 times, after which samples were blocked in Buffer Solution containing 5%BSA for 45 minutes. Samples were then washed again with buffer solution before incubation with the primary rabbit anti-human IK_{Ca}, or SK_{Ca} antibodies ((clones: M20 and M75, respectively), 1:200), or with blocking peptides in the buffer solution for 1 hour.[151, 152] Samples were washed again, and then incubated with secondary antibody using the inverted coverslip on droplet technique - donkey anti-rabbit AlexaFluor 488 (1:200), for 1 hour. Samples stained for F-actin, were washed again before incubation with phalloidin-AlexaFluor 568 (1:40), for 30 minutes again using the inverted coverslip on droplet method. For the samples not stained with phalloidin, this step was skipped. Samples were further washed with buffer solution and then mounted using ProLong Diamond AntiFade Mountant (5µl) onto microscope slides and subsequently allowed to cure for 24 hours before sealing. Images were captured using magnifications of 100x, and 5 separate fields of view per sample, on the Leica TCS SP5 confocal microscope (Leica Microsystems, Concord, ON, Canada).

To use the blocking peptides for the IK_{Ca} and SK_{Ca} antibodies, the peptides were incubated with the antibodies the day before staining, following a previously published protocol. The

blocking peptides (10µl) were incubated for 1 hour at room temperature with the corresponding antibody (10µl), incubation continued overnight at 4ºC. The combination of blocking peptides with the antibodies was treated as any other primary antibody used, as described above.

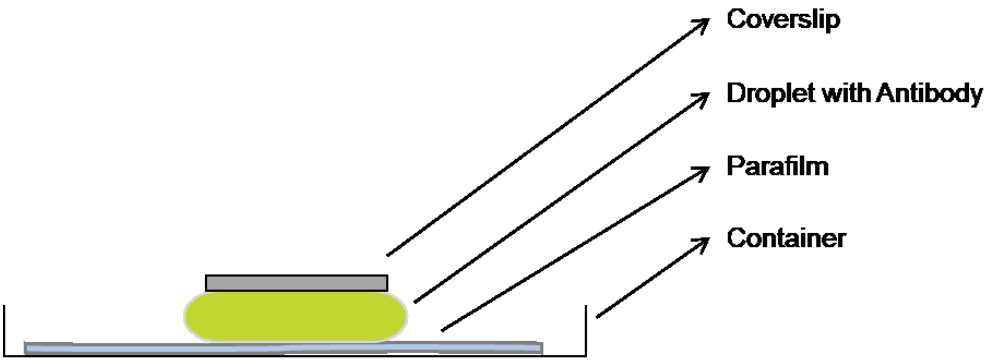

Figure 4. Inverted Coverslip on Droplet Microscopy Method.

3.4 Aggregometry

To determine the extent of platelet aggregation when in the presence of the K_{Ca} agonists, a Chrono-Log Model 560 Dual Channel Lumi-Aggregometer (Havertown, PA, USA) was used. Aliquots (500µl-1ml) of isolated platelets ($2.5x10^8$/ml) were added to cuvettes and placed in the aggregometer with a stir bar rotating at 900 rpm. To induce platelet aggregation, platelet agonists (collagen (1µg/ml) or thrombin (0.1U/ml)) were added after a 2-minute equilibration period. The K_{Ca} channel activators or DMSO vehicle control (0.1% DMSO in saline) were added at time 0 of the experiment. In some experiments, the addition of the K_{Ca} channel inhibitors, or L-arginine to the platelets also occurred at time 0 (Figure 5). Platelet aggregation was reported as a percent increase in light transmittance. In some experiments, result were expressed as percent maximal aggregation.

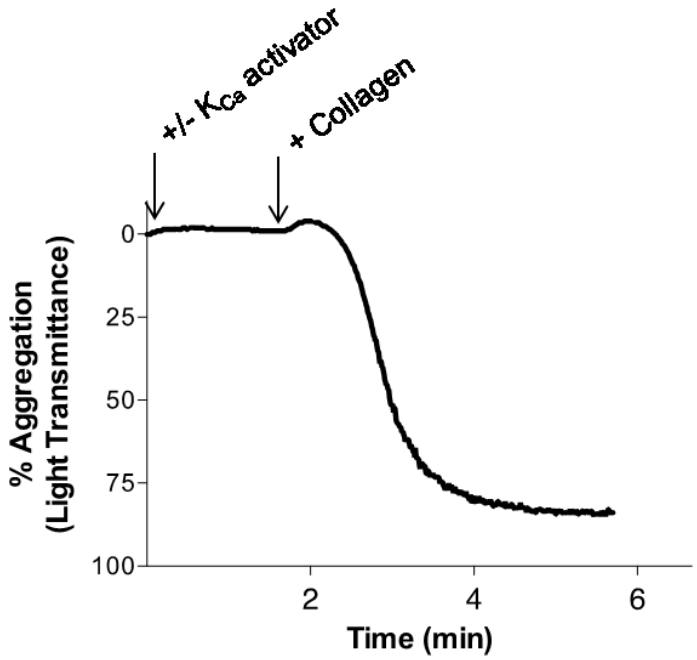

Figure 5. Aggregometry Experimental Design. Addition of any compound, including the K_{Ca} activators, or inhibitors, vehicle control or L-arginine occurred at time 0. Collagen or thrombin were added at the 2-minute time point to induce activation and shape change, followed by aggregation, measured as an increase in light transmittance.

3.5 Q-sense Quartz Crystal Microbalance Flow Model of Platelet Adhesion and Aggregation

To assess platelets in a more physiological relevant model of laminar flow, the Q-sense™ Quartz Crystal Microbalance (QCM) was used (Biolin Scientific, Gothenburg, Sweden) Santos-Martinez et al.[153] The QCM flow system can detect nanogram changes in mass based on change in the oscillation frequency of quartz crystals with an electric field applied to them. An increase in mass as a result of adhering platelets on a collagen-coated quartz crystal sensor causes the crystal oscillation frequency, *f*, to decrease. In addition, the QCM system measure the quartz crystal's energy dissipation providing information with regards to the rigidity/thickness of the bound platelet layer. In brief, gold-coated quartz-crystal sensors were coated with collagen (10µg/ml) for 1 hour and allowed to air dry. PBS and Tyrode's buffers were degassed in a sonicating water bath (VWR International, Mississauga, ON, Canada) kept at 37°C. The degassed PBS was then flowed through the tubing system of the QCM for 30 minutes, with coated sensors in place, to equilibrate the QCM. Isolated platelets were resuspended at $2.0x10^8$ platelets/ml using the degassed Tyrode's buffer, and allowed to rest in a heating block set to 37°C. This platelet concentration was previously determined to be effective and was chosen to allow a controlled stream of platelets and eliminate the likelihood of potential aggregate buildup within the QCM tubing. Platelets were incubated with either vehicle, SKA-31 (10µM) or CyPPA(10µM), and allowed to flow through the QCM system to the sensors at 0.1ml/minute over a time period of 1.5 hours and frequency and energy dissipation were recorded. To further analyze the adhered platelet layer, after the 1.5-hour recording period, sensors were fixed in 4% paraformaldehyde for 20 minutes. Sensors were then washed 3 times with 1ml of PBS, and permeabilized using 0.1% Triton X-100 in PBS for 10 minutes. The Triton solution was then rinsed out with PBS, and platelets were then stained for F-actin using Phalloidin-AlexaFluor-568 (1:40) for 20 minutes. Sensors were attached to microscope

slides using nail polish, then covered with ProLong Diamond AntiFade Mountant (5µl), after which coverslips were affixed on top of the sensors and to microscope slides. Samples were allowed to set overnight and slides were sealed the next day. Sensors were imaged using the Leica TCS SP5 confocal microscope, using the HeNe 543 laser. Z-stacks were taken every 0.3µm at 63x magnification, and were assembled into max projections, using pre-sets built into the Leica LAS AF software (Leica Microsystems, Concord, ON, Canada), of the entire aggregate within the same plane.

3.6 Measurement of Platelet NO Generation by DAF-FM Diacetate

DAF-FM diacetate (4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate) is a fluorescent dye often used to detect/quantify low concentrations of NO. The dye is very weakly fluorescent, until it reacts with NO to form a benzotriazole with an almost 160 fold increase in fluorescence.[154] Platelets were loaded with DAF-FM diacetate according to a previously established protocol.[77] Briefly, DAF-FM diacetate (10μM) was incubated in the PRP for 30 minutes in the dark. Subsequently, platelet isolation continued as described in Section 3.2, with addition of PGI_2 followed by a 900g centrifugation, removal of PPP, washing of the platelet pellet 3 times with 1ml Tyrode's buffer and resuspension of the DAF-FM loaded platelets at a concentration of $2.5x10^8$ platelets/ml. This was followed by a 1-hour benchtop resting period in which the platelet inhibitory effects of PGI_2 were allowed to wear off.

Aggregation of DAF-FM stained platelets was carried out as described in Section 3.4. Subsequently, 10μl aliquots of platelets from the aggregometer were then collected at the end of the aggregation (time point: 6 minutes), and incubated with 10 μl of anti-CD42b-PE antibody with 80μl of saline – for a total volume of 100 μl – for 15 minutes in the dark. Samples were then topped up to 1ml with saline and platelet DAF-FM fluorescence measured from the 25,000 events collected using a Beckman Coulter Quanta SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA) as described previously.[77]

3.7 Changes in Platelet Calcium Signalling

Intracellular calcium signalling measurements were made using Fluo-4 AM-stained platelets. Fluo-4 AM, is a cell-permeant calcium indicator that exhibits increased fluorescence upon Ca^{2+} binding. Fluo-4 AM (2.5μM) was incubated in the PRP for 30 minutes, followed by isolation of platelets and aggregometry as described in Section 3.6.

For initial protocol optimization and to determine the point in which maximal Ca^{2+} signalling occurs during aggregation, aliquots (10μl) of Fluo-4AM stained platelets were sampled at time 0, 1-minute, maximum shape change, 50% aggregation and at 6 minutes. These aliquots were then stained for CD42b using 10μl anti-CD42b-PE, with 80μl saline, for 5 minutes in the dark and then topped up to 1ml with saline. Fluo-4 AM fluorescence was determined by a previously established flow cytometry protocol. Platelets were identified as CD42b positive events on FL2 and then gated to determine Fluo-4 AM fluorescence on the FL1 detector, and 25,000 events collected. Maximal Fluo-4 AM fluorescence was detected at the 50% aggregation time point and subsequent experiments measuring the effects of SKA-31 or CyPPA on changes in platelet Ca^{2+} signalling were performed comparing Fluo-4 AM fluorescence at this point to that of maximum shape change.

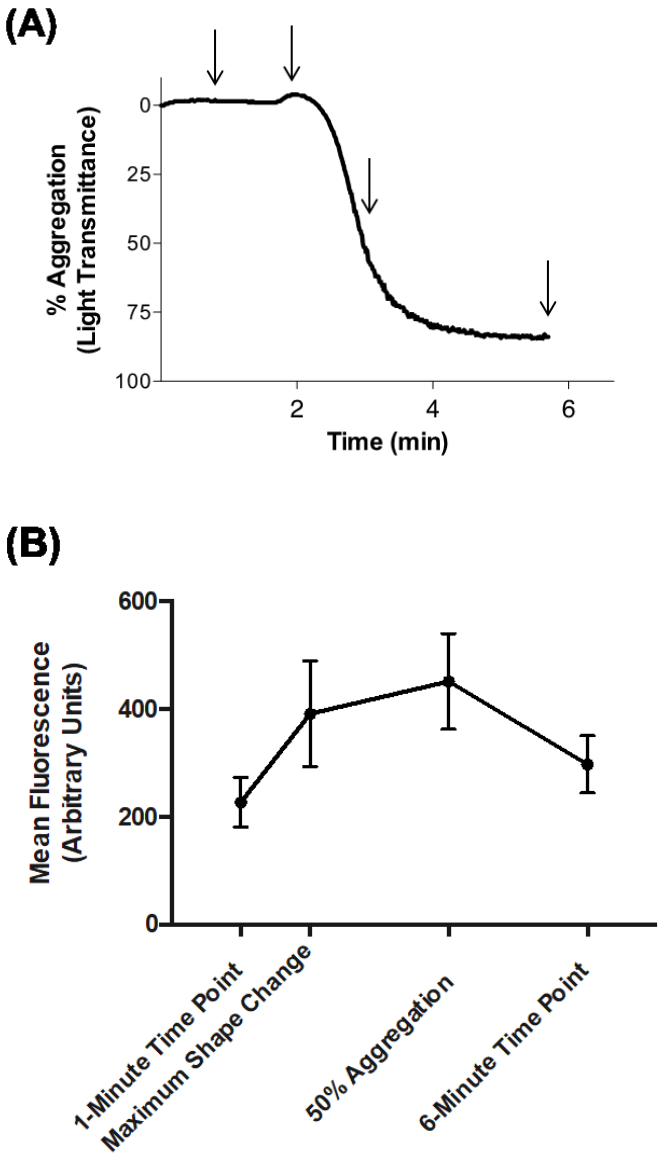

Figure 6. Calcium-signalling experimental design. (A) Typical aggregometry trace, in response to collagen, arrows depict the time points of aliquot collection which are then measured for calcium (B).

3.8 Measurement of ATP Release by Chemiluminescence

ATP release from platelets was measured as a marker of dense granule secretion.[155] 50µl ChronoLume™ Luciferin/Luciferase (Havertown, PA, USA) reagent (440 luciferase units/ml and 4 mg/ml of luciferin) was added to a cuvette filled with 450µl of isolated platelets and placed in the lumi-aggregometer. The chemiluminescence signal was then allowed to equilibrate and reach steady baseline – for experimental purposes considered to be time 0. Vehicle control, SKA-31 or CyPPA were then added to the platelet filled cuvette. Two minutes later collagen (1µg/ml) was added to stimulate platelet aggregation and granule secretion, and luminescence was measure by the lumi-aggregometer. Measurements in amount of ATP secreted from platelets varied widely between individuals and therefore data was normalized to vehicle controls for each individual and expressed as a percentage of maximal release.

3.9 P-selectin Flow Cytometry as a Marker of Alpha Granule Secretion

P-selectin measurements were used as a marker of alpha granule secretion, as P-selectin is stored in alpha granules and is exposed to the platelet membrane surface upon granule secretion. Platelets were isolated and aggregometry was performed as described in sections 3.2 and 3.4. At the 6-minute time point of aggregometry, 10µl aliquots of platelets were collected and incubated with 10µl anti-CD62P-PE (1:10) and 80 µl saline, for a total volume of 100 µl, for 15 minutes in the dark. Samples were then topped up to 1ml with saline and run on the Beckman Coulter Quanta SC MPL flow cytometer using a protocol previously established for P-selectin measurements in platelets.[155] P-selectin surface exposure was measured by mean fluorescence on detector FL2, and 10,000 events collected.

3.10 Statistical Analyses

All statistical analyses were performed using GraphPad Prism 6 software. Results are presented as mean ± standard error of the mean, where the N reported is representative of each individual blood donor used in a given experiment. Comparison of multiple groups to each other or to a control group were performed using repeated measures one-way analysis of variance (ANOVA) with either Tukey or Dunnet post-hoc test, where appropriate, and as indicated. P-values of less than 0.05 were considered to be statistically significant.

4. RESULTS

4.1 Detection and Visualization of K_{Ca} Channel Subtypes and Distribution in Platelets

Previous studies have determined the presence of K_{Ca} channels in platelets using fluorescent probes and electrophysiological methods.[87, 115] These studies suggest that the subtype most likely to be present are IK_{Ca} at a very low number of channels, approximately 5-7 per platelet, based on size of conductance recorded as well as apamin (SK_{Ca} channel blocker) insensitivity.[87, 115] The first objective of this study was to confirm the presence of the K_{Ca} channels present in platelets, which was investigated using confocal microscopy of platelets stained with IK_{Ca} and SK_{Ca} channel antibodies (clones: M20 and M75, respectively).[151] Figure 7 demonstrates the presence of IK_{Ca} channels (B), as well as SK_{Ca} channels (D). The use of blocking peptides in combination with the antibodies demonstrates an inhibition in the fluorescence signal for IK_{Ca} channels (C), and SK_{Ca} channels (E).

The second portion of objective one was to determine the localization of the K_{Ca} channels within the platelet. Again, confocal microscopy was used, but samples were co-stained with phalloidin to visualize F-actin. The use of phalloidin allowed for visualization of the platelet membrane and subsequent determination of the location of K_{Ca} channels within the platelet (Figure 8). Panels D-F, demonstrate the F-actin fluorescence in red, and panels G-I show the overlap of green and red for each respective sample. The green fluorescence confirms presence of IK_{Ca} channels in platelets, but the detection of SK_{Ca} channels is lost. However, only two platelet donors out of the total five displayed any fluorescence for the SK_{Ca} channel. When examining the overlaid images (G-I), there is some co-localization between green and red fluorescence, suggesting the IK_{Ca} channels (green) are localized within the platelet membrane (red).

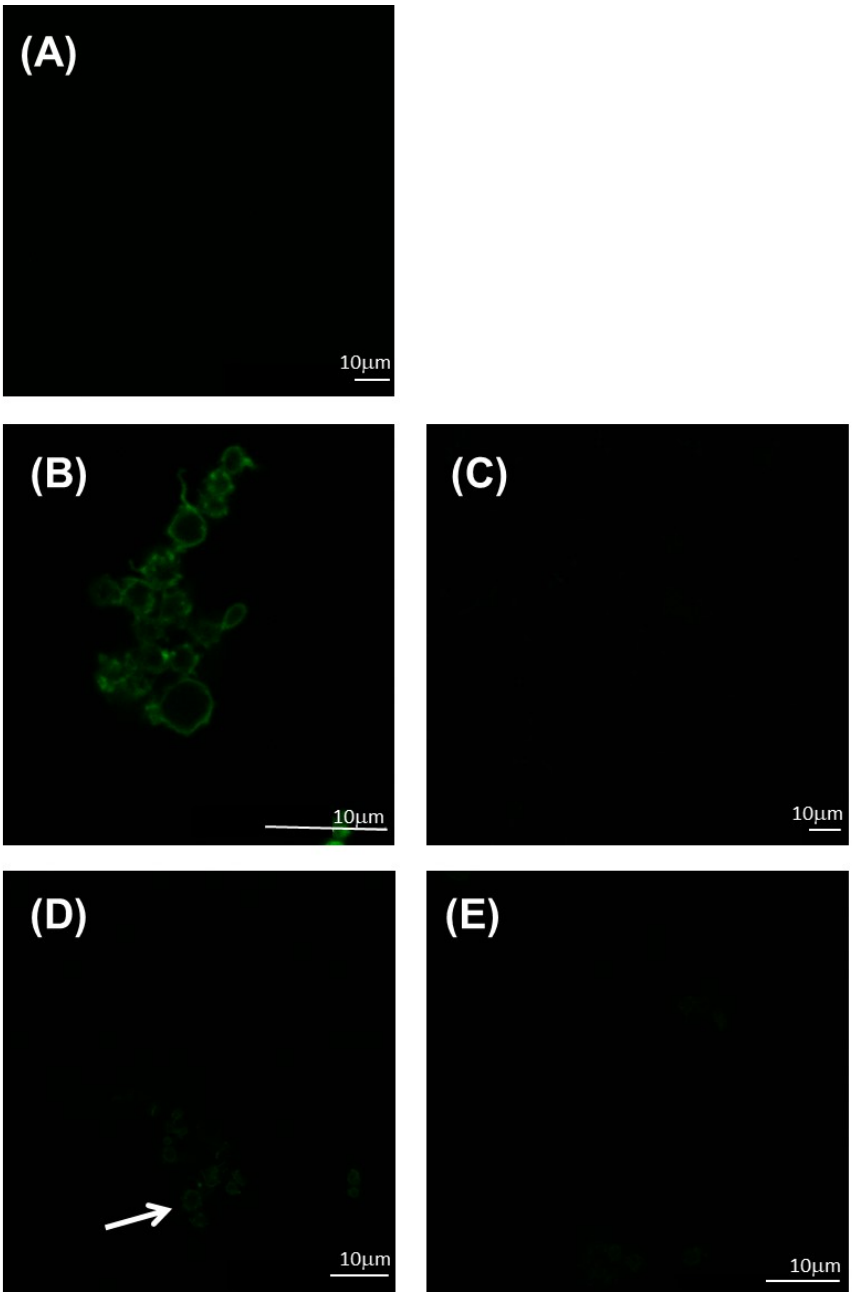

Figure 7. Representative confocal microscopy of IK_{Ca} and SK_{Ca} channels in platelets.

Confocal microscopy images of platelets (1000x) stained for (A) IgG, (B) IK_{Ca} channels, (C) IK_{Ca} channels incubated with blocking peptide, (D) SK_{Ca} channels (arrows) and (E) SK_{Ca} channels incubated with blocking peptide. Representative image of total N=5.

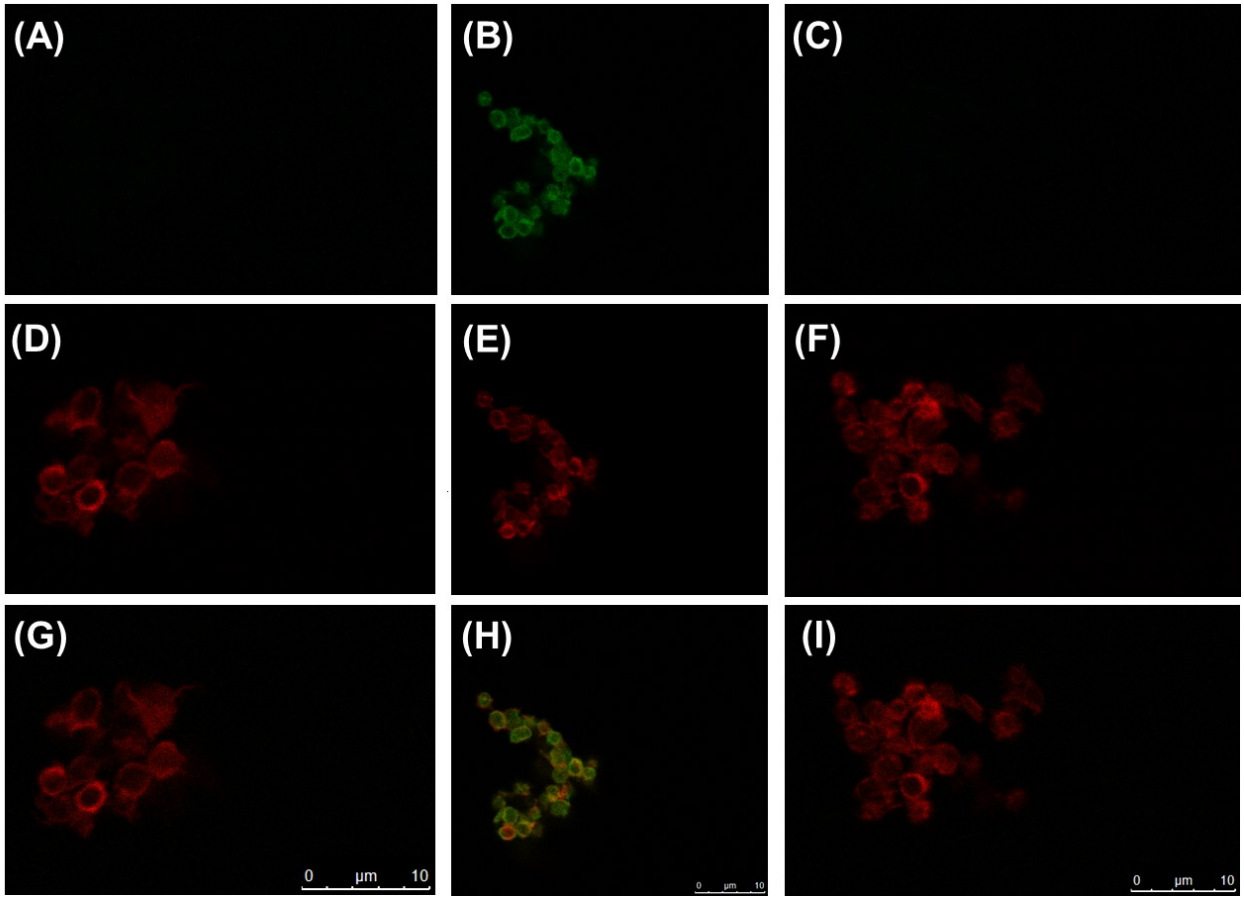

Figure 8. Representative confocal microscopy of distribution of K_{Ca} channels in platelets. Confocal microscopy images of platelets (1000x) stained for (A) IgG control, (B) IK_{Ca} channels, or (C) SK_{Ca} channels, in green. Co-stained with phalloidin, in red, for (D) IgG control, (E) IK_{Ca} channels, or (F) SK_{Ca} channels to visualize F-actin and thus the platelet membrane. Overlay images for (G) IgG control (H) IK_{Ca} channels and (I) SK_{Ca} channels, demonstrate the localization of the channels in relation to the platelet membrane. Representative image of total N=5.

4.2 Inhibition of Platelet Aggregation by K_{Ca} Channel Activation

In order to determine the effects of K_{Ca} channel activation on platelet function, aggregometry with pharmacological activators of K_{Ca} channels was performed.

The IK_{Ca} and SK_{Ca} channel activators used were SKA-31 and CyPPA, respectively, which were added at the beginning of aggregation (time 0) and allowed to equilibrate for 2 minutes before addition of the platelet agonist, collagen (1μg/ml), with data collection occurring until the 6-minute mark. The results demonstrate concentration-dependent inhibition of collagen-induced aggregation of platelets, with SKA-31 (Figure 8) and CyPPA (Figure 9). SKA-31 demonstrates an approximate 50% inhibition at 10 μM, and complete inhibition of platelet aggregation at 100 μM. CyPPA demonstrated 40% inhibition of aggregation at the 10 μM concentration, and complete inhibition of platelet aggregation at 100 μM.

The 10 μM concentration of the K_{Ca} channel activators was chosen for use in further experiments, as it demonstrated approximate 50% inhibition in maximal aggregation to 1 μg/ml of collagen.

In contrast, when using thrombin (0.1U/ml) as the platelet agonist however, there was no significant difference between groups (Figure 10). However, only the 10 μM concentration of SKA-31 and CyPPA were used, and no concentration response curve was generated.

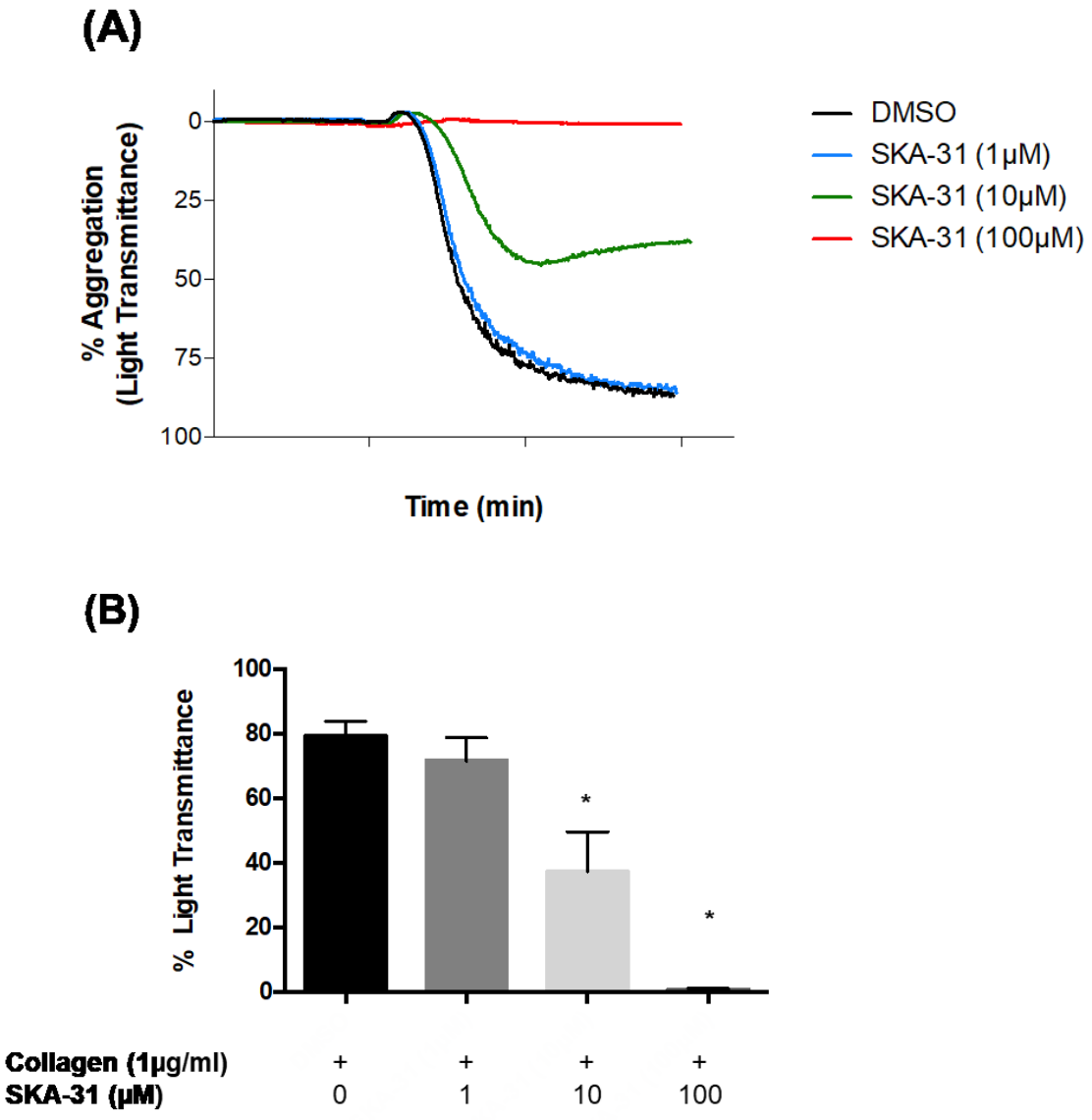

Figure 9. Concentration dependent inhibition of platelet aggregation in response to IK_{Ca} channel activation. (A) Representative aggregometry traces and (B) concentration response experiments of platelets incubated with increasing concentrations of the IK_{Ca} channel activator, SKA-31, and stimulated to aggregate by collagen (1µg/ml). Statistics: RM one-way ANOVA with Tukey post-hoc test. N=5. *P<0.05 to vehicle control.

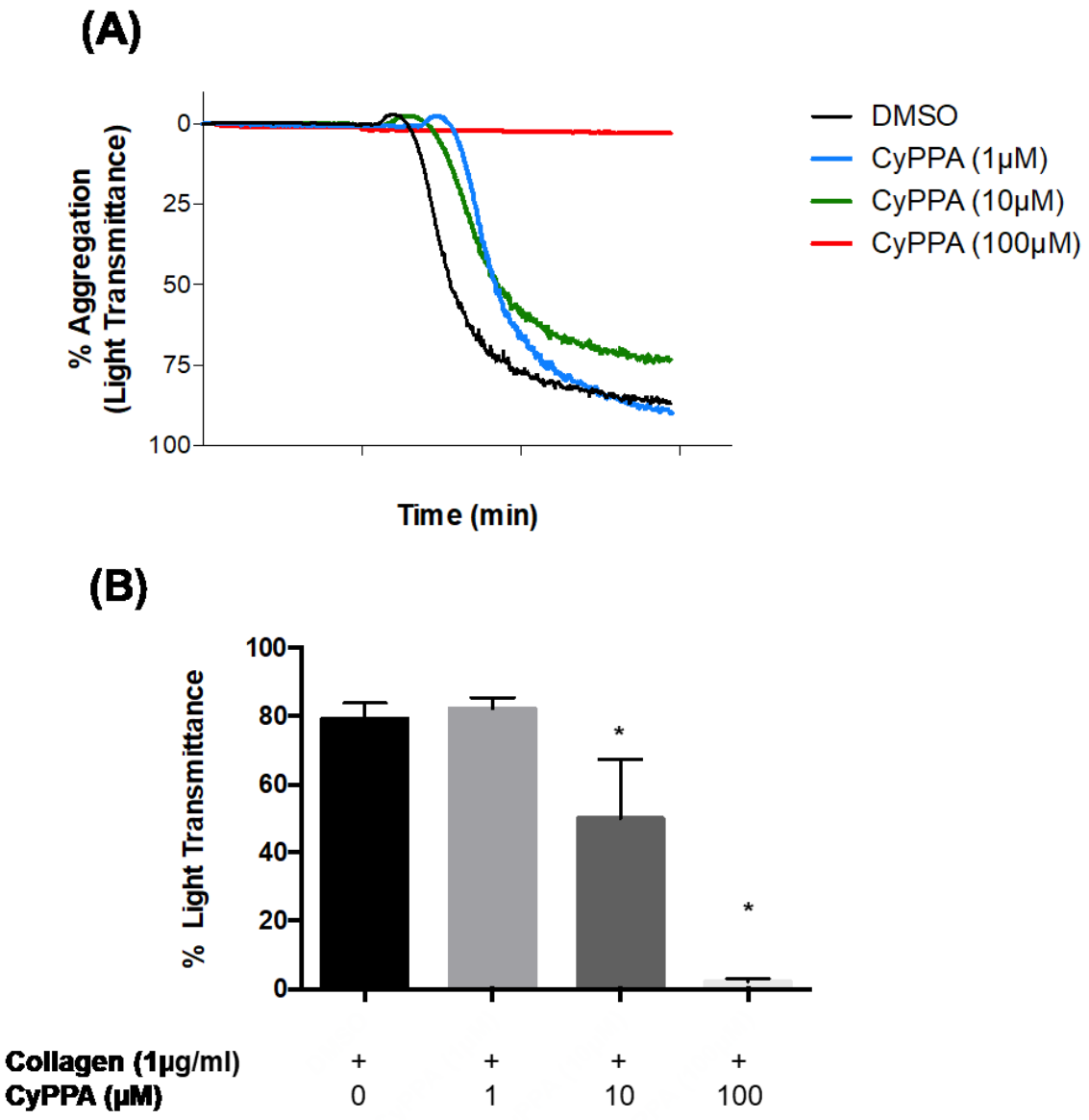

Figure 10. Concentration dependent inhibition of platelet aggregation in response to SK_{Ca} channel activation. (A) Representative aggregometry traces and (B) concentration response experiments of platelets incubated with increasing concentrations of the SK_{Ca} channel activator, CyPPA, and stimulated to aggregate by collagen (1µg/ml). Statistics: RM one-way ANOVA with Tukey post-hoc test. N=5. *P<0.05 to vehicle control.

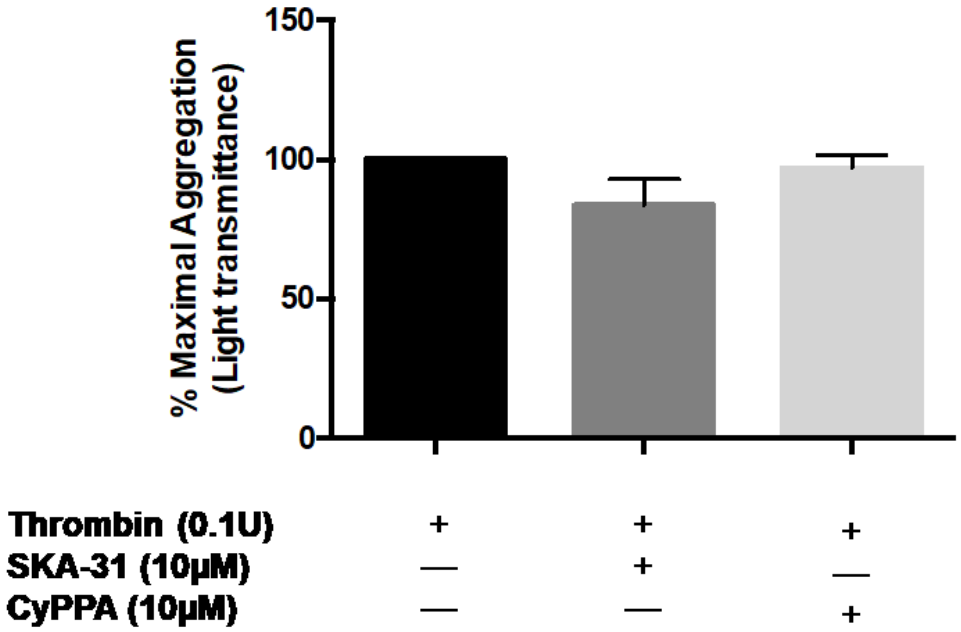

Figure 11. Aggregation response to thrombin. Summary data of the effects of SKA-31 (10 μM) and CyPPA (10 μM) on platelet aggregation induced by thrombin (0.1 U/ml). Statistics: RM one-way ANOVA using Dunnett post-hoc test. N=5. *P<0.05 when compared to vehicle control.

To confirm selectivity of SKA-31 and CyPPA for activation of IK_{Ca} and SK_{Ca} channels, pharmacological channel inhibitors (TRAM-34 for IK_{Ca} channels (K_{Ca} 3.1) and apamin for SK_{Ca} channels (K_{Ca} 2) were used in combination with the activators in platelet aggregation experiments. The inhibitors are reported to have a higher selectivity for the channels, the K_d for TRAM-34 is 20nM and has been reported in the 10-30pM range for apamin, making them good tools to use for this purpose.[156, 157]

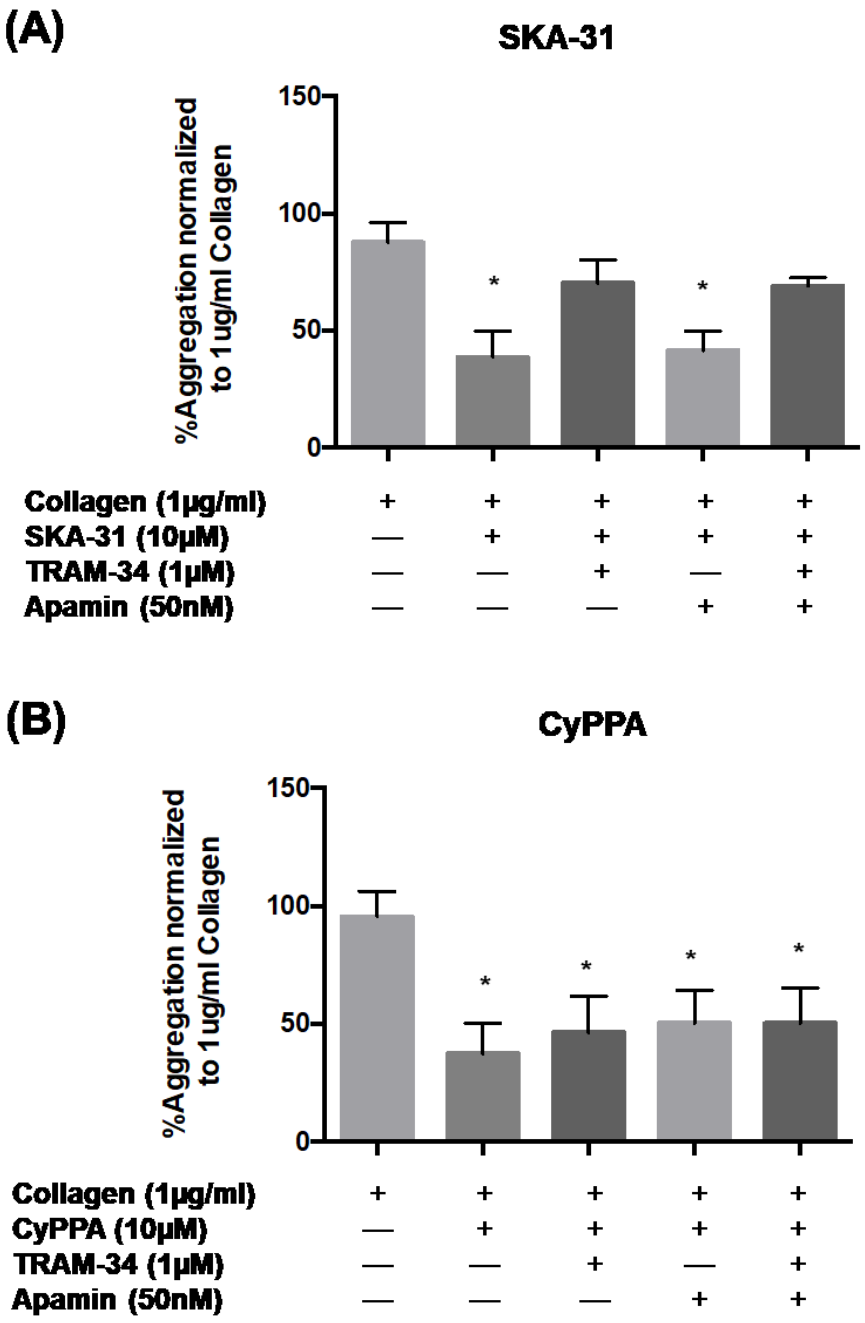

Figure 12. Pharmacological confirmation of platelet IK_{Ca} activation by SKA-31. Pharmacological confirmation of action of (A) SKA-31, but not for (B) CyPPA, by use in combination with the respective channel blockers, TRAM-34 (IK_{Ca} channel antagonist) and apamin (SK_{Ca} channel antagonist). Statistics: RM one-way ANOVA with Dunnett post-hoc test. N=5. *P<0.05 to vehicle control.

To study the role of IK_{Ca} or SK_{Ca} channels in platelet aggregation, in the absence of pharmacological activation, the effects of K_{Ca} channel blockers, TRAM-34 and apamin, alone were studied. The platelets for these experiments were stimulated using a lower collagen concentration of 0.6 μg/ml, this lower concentration was used in order to allow for detection of results that may either potentiation or inhibition of aggregation. The results in Figure 13 demonstrate a non-significant effect of TRAM-34 on platelet aggregation, whereas apamin was able to significantly inhibit platelet aggregation by approximately 30%. These results have implications in the interpretation of the effects of CyPPA not being reversed by apamin, Figure 12. This may also provide explanation of why apamin, who inhibits aggregation, is not able to reverse the inhibition induced by CyPPA.

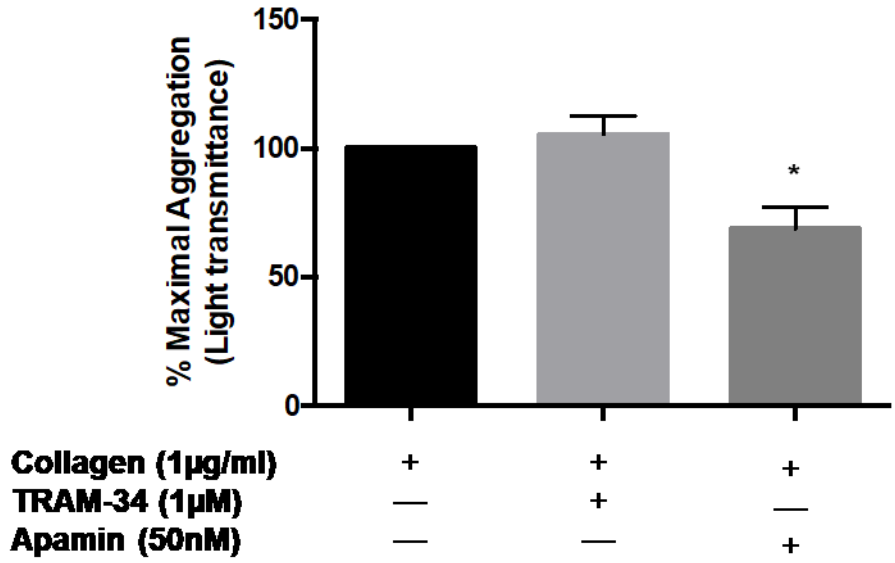

Figure 13. Platelet aggregation is inhibited by SK_{Ca} channel inhibitor, Apamin. Response of platelets to the IK_{Ca} channel antagonist, TRAM-34 (1µM) alone or SK_{Ca} channel antagonist, apamin (50nM) alone, when stimulated with 0.6µg/ml collagen. Statistics: RM one-way ANOVA with Dunnett post-hoc test. N=5. *P<0.05 to vehicle control.

4.3 Effects of K_{Ca} Channel Activation Under Laminar Flow

Although aggregometry is an excellent model for studying platelet aggregation, simply by design it creates a small vortex, and is therefore perhaps not the most physiological model. *In vivo*, however other than certain disease states, turbulent flow is not often observed, and laminar flow is therefore a more physiological and sensitive method.[158] Thus, to assess the effects of the K_{Ca} channel activators in a model of laminar flow, a previously-developed novel technique, the Q-sense™ QCM was used.[153]

Since the quartz crystals in the sensors oscillate in an electric field, the detectors measure frequency and dissipation of the sensors over time. Frequency is the measurement associated with changes in mass, an increase in mass bound to the sensor's surface causes the crystal's oscillation frequency to decrease. The Sauerbrey equation is what is used to measure a change in mass as a function of change in frequency, but becomes invalid if the buildup does not remain rigid throughout the experiment, ultimately underestimating the mass of the buildup. Dissipation takes this account, and in the event of a viscoelastic film, modifies the equation in order to provide a more accurate measure of mass of the buildup. Therefore the additional ability of the Q-sense™ QCM to measure dissipation alongside frequency, is superior to measuring frequency alone and provides more information on the rigidity of buildup on the sensor. In regards to studying platelets, it has been determined that the measure of frequency corresponds to platelet adhesion to the sensors, whereas dissipation measures the rigidity and thickness of the film built up on the sensor, largely corresponding to the thickness of aggregated platelets.[153]

In these sets of experiments, none of the treatments significantly affected the respective changes in frequency measured (Figure 14B). However, dissipation was significantly inhibited by activation of the IK_{Ca} channels in platelets by SKA-31 (Figure 14C). A decrease in dissipation

with no significant difference in frequency when compared to control is suggestive of inhibited platelet aggregation but not platelet adhesion. Figure 15 displays the images obtained from the sensors as maximum projections of Z-stacks taken at every 0.3μm. The microscopy revealed that under flow conditions IK_{Ca} channel activation with SKA-31 inhibits aggregate formation, but not adhesion of platelets, as suggested by the frequency and dissipation measurements. This also corroborates the inhibition of platelet aggregation detected in the aggregometer upon IK_{Ca} activation. CyPPA did not significantly inhibit frequency or dissipation, however the microscopy may demonstrate buildup of smaller aggregates, therefore still somewhat inhibiting platelet aggregation under laminar flow.

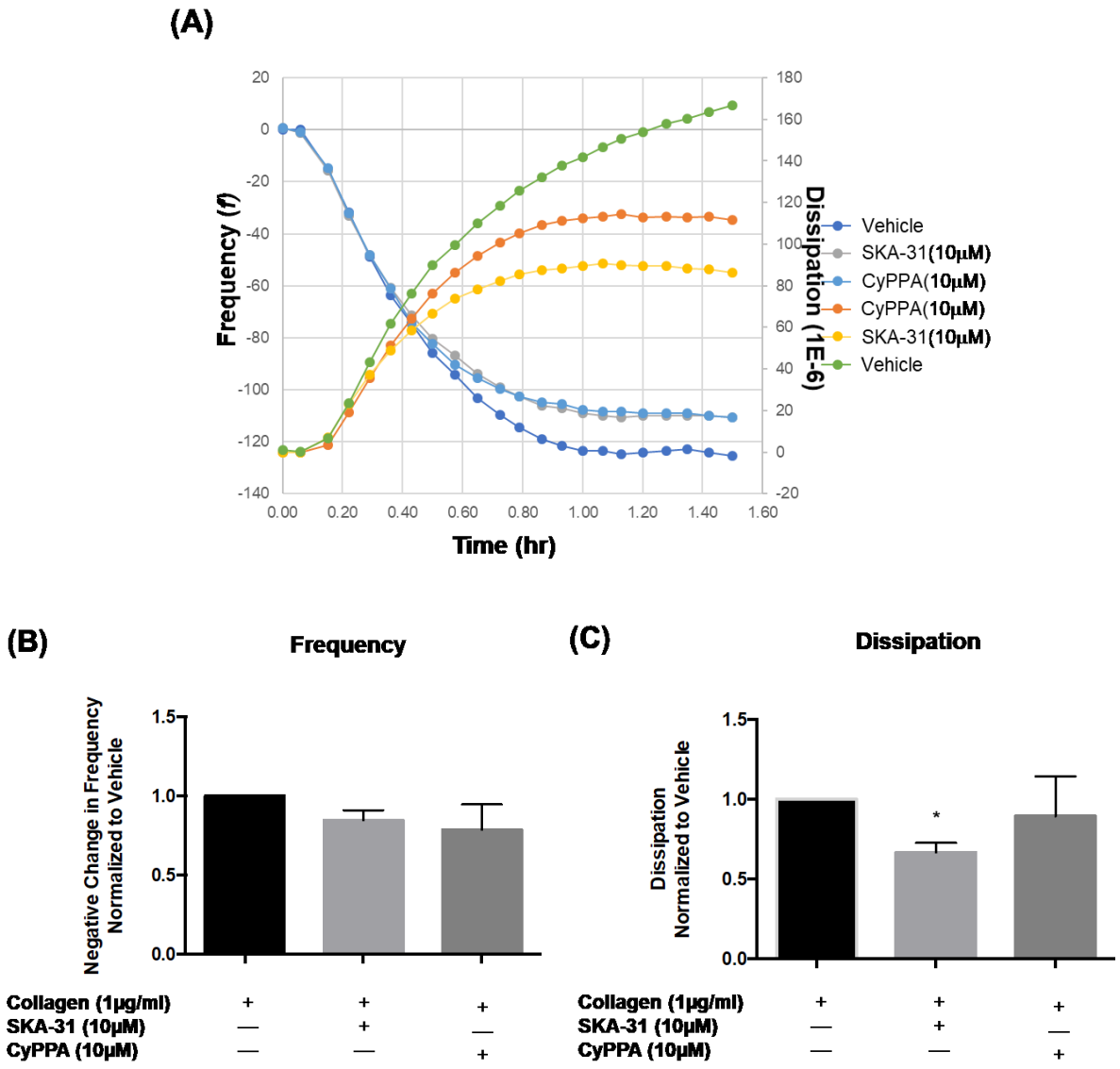

Figure 14. QCM laminar flow model demonstrates inhibition of aggregation with IKCa channel activation. (A) Representative frequency (top) and dissipation (bottom) traces generated by the Q-Sense™ QCM. (B) Quantified and normalized frequency and (C) dissipation values. Statistics: RM one-way ANOVA with Dunnett post-hoc test. N=4. *P<0.05 to vehicle control.

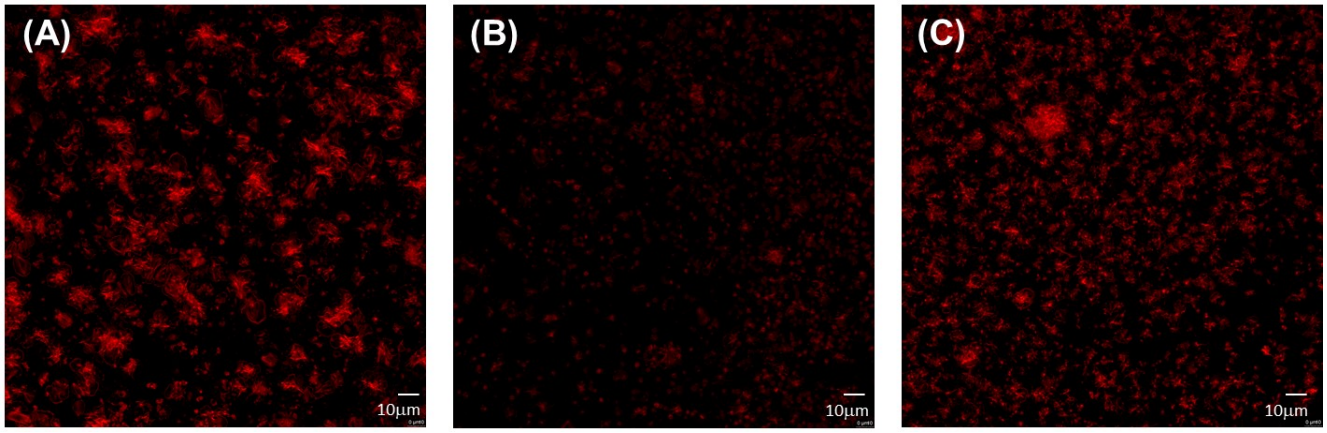

Figure 15. Representative confocal microscopy of adherent platelets from QCM sensors. Confocal microscopy images (max projections of z-stacks) taken at 63x of platelets that have adhered to QCM sensors. Platelets are stained for F-actin (red), and exposed to either (A) vehicle control, (B) SKA-31, or (C) CyPPA, before they pass through the sensors coated with collagen (10µg/ml). Representative of N=4.

4.4 Activation of IK_{Ca} and SK_{Ca} Channels Inhibits Platelet-NO Generation

The major hypothesis of this study was that activation of IK_{Ca} or SK_{Ca} channels would increase platelet-NO generation, and thus provide a mechanism to enhance this endogenous negative feedback pathway. This hypothesis is based on how the IK_{Ca} and SK_{Ca} channels function in endothelial cells. In endothelial cells, an increase in $[Ca^{2+}]_i$ results in activation of eNOS and of IK_{Ca} and SK_{Ca} channels. Activation of the channels results in K^+ efflux and membrane hyperpolarization, leading to Ca^{2+} entry via TRPC channels, thereby feeding-forward to generate additional NO via stimulation of eNOS.

NO generation by platelets was assessed using DAF-FM diacetate staining. DAF-FM diacetate is a cell-permeable dye that binds selectively to NO and is weakly fluorescent in its unbound form, but upon binding to NO forms a benzotriazole and becomes 160 times more fluorescent.[154]. Once inside the cell, DAF-FM diacetate is cleaved by esterases and becomes cell-impermeant, trapping the dye inside the cell.

As previously described, aggregation of platelets with collagen in the presence of L-arginine resulted in increased platelet NO production as measured by DAF-FM fluorescence.[77] Surprisingly, SKA-31 (10μM) or CyPPA (10μM) incubation caused an unexpected but significant decrease in mean fluorescence. This translates into a significant inhibition of NO generation, as a result of activation of either IK_{Ca} or of SK_{Ca} channels, back to almost basal, resting platelet levels.

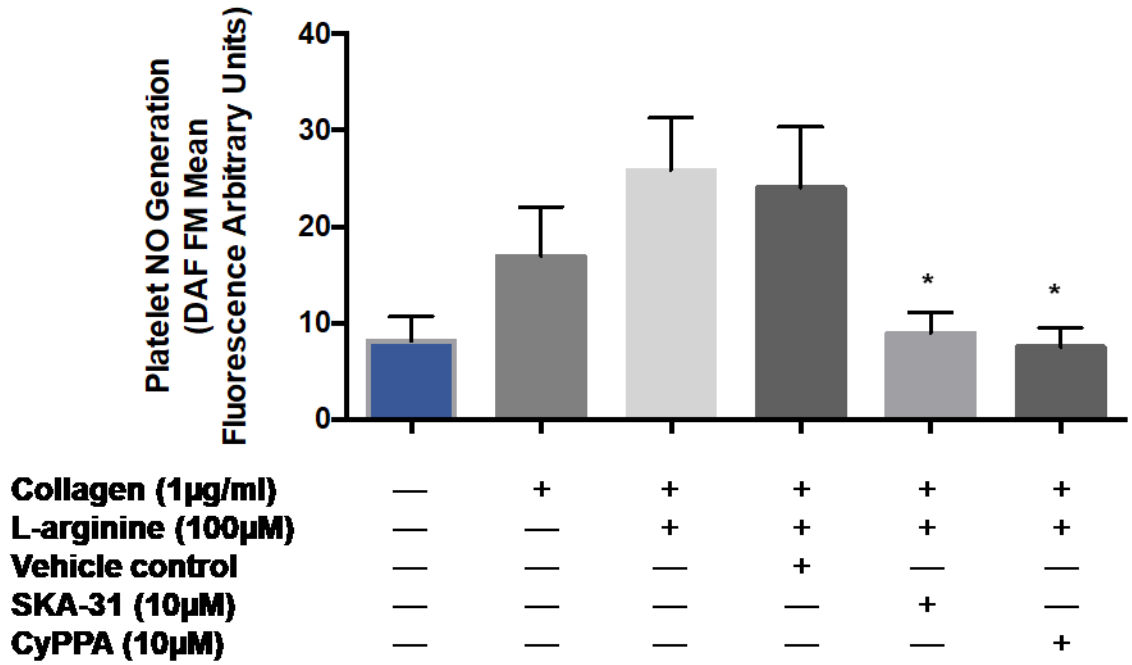

Figure 16. Platelet NO generation inhibited by IK_{Ca} and SK_{Ca} channel activation. NO generation of platelets as measured using DAF-FM diacetate staining and flow cytometry. Statistics: RM one-way ANOVA with Dunnett post-hoc test. N=5. *P<0.05 to vehicle control.

4.5 K_{Ca} Channel Activation Inhibits Intracellular Calcium Signalling in Platelets

In endothelial cells, the main driving force for the activation of IK_{Ca} and SK_{Ca} channels is an increase in $[Ca^{2+}]_i$, either from intracellular store release or by influx via TRPC channels, as a result of the IK_{Ca} and SK_{Ca} channel activation. The initial hypothesis thus, was that a localized increase in calcium levels, as a result of membrane hyperpolarization and Ca^{2+} entry, would stimulate generation of NO via eNOS and inhibit platelet aggregation. However, as K_{Ca} channel activation inhibited both platelet aggregation and NO generation, both of which are Ca^{2+}-dependent processes, the Ca^{2+}-signalling in platelets became relevant to study.

Relative calcium levels were assessed by using Fluo-4 AM stained platelets following aggregometry. The results obtained by Fluo-4 AM aggregometry/flow cytometry, demonstrate a significant decrease in calcium fluorescence when exposed to the IK_{Ca} channel activator, SKA-31 but not SK_{Ca} channel activator, CyPPA, as compared to vehicle control responses.

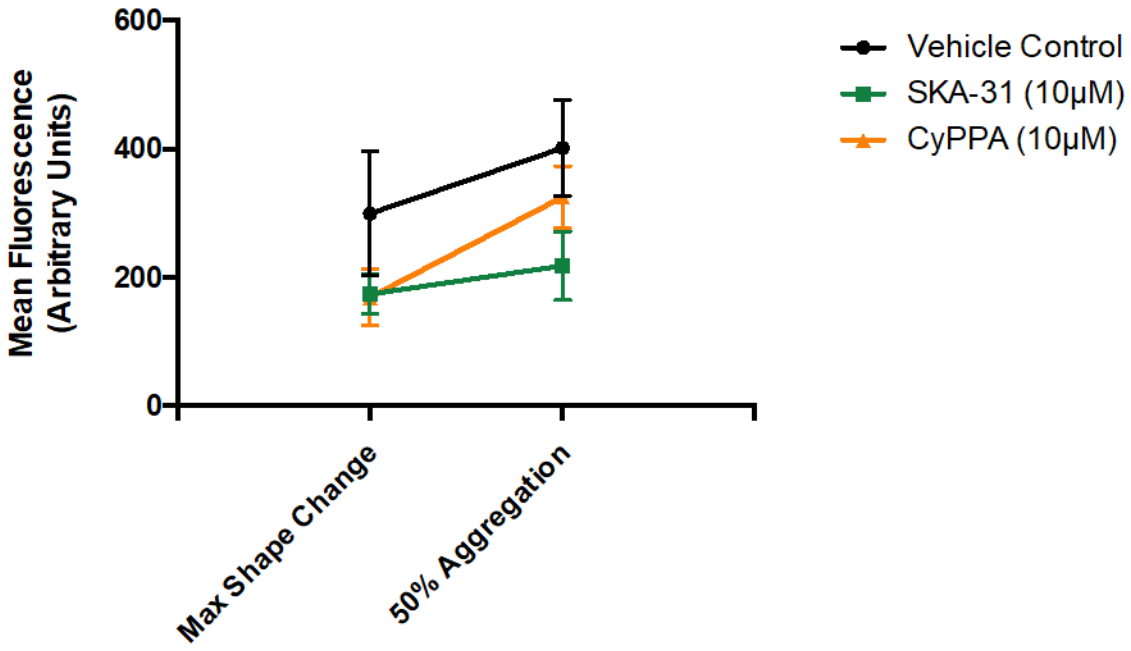

Figure 17. Changes in calcium signalling of platelets treated with K_{Ca} channel agonists. Changes in the calcium signalling response of platelets to IK_{Ca} or SK_{Ca} treatment, demonstrated a decrease in calcium with treatment. Statistics: RM two-way ANOVA with Sidak post-hoc test. N=5. P=0.0441 for effect of IK_{Ca} treatment, but not time.

4.6 K_{Ca} Channel Activation Inhibits Platelet Granule Secretion

Since Ca^{2+}-signalling was inhibited in platelets, providing an explanation for the inhibition of aggregation and unexpected inhibition of eNOS, other calcium-dependent processes: dense and alpha granule secretion were also investigated. Other mechanisms involved in the platelet aggregatory machinery were investigated, such as dense and alpha granule secretion. Granules are secreted upon activation of platelets as a result of increasing $[Ca^{2+}]_i$, and due to factors released, act as feed forward mechanisms in the aggregation cascade, to aide in recruiting nearby platelets.

As a marker of dense granule secretion, ATP release by platelets was measured by chemiluminescence. The measurements demonstrate inhibition of dense granule secretion by SKA-31 and CyPPA, when compared to vehicle control, Figure 18A.

Alpha granule secretion was investigated using flow cytometry of the marker P-selectin. P-selectin is not found on the surface of unactivated platelets, but is stored in the alpha granules. Upon activation and granule secretion, the P-selectin expression on the platelet surface becomes highly abundant. Figure 18B demonstrates that SKA-31 significantly inhibited P-selectin expression, where in contrast CyPPA did not significantly inhibit P-selectin surface exposure compared to the vehicle control. It is unknown why CyPPA treatment would not inhibit alpha granule secretion.

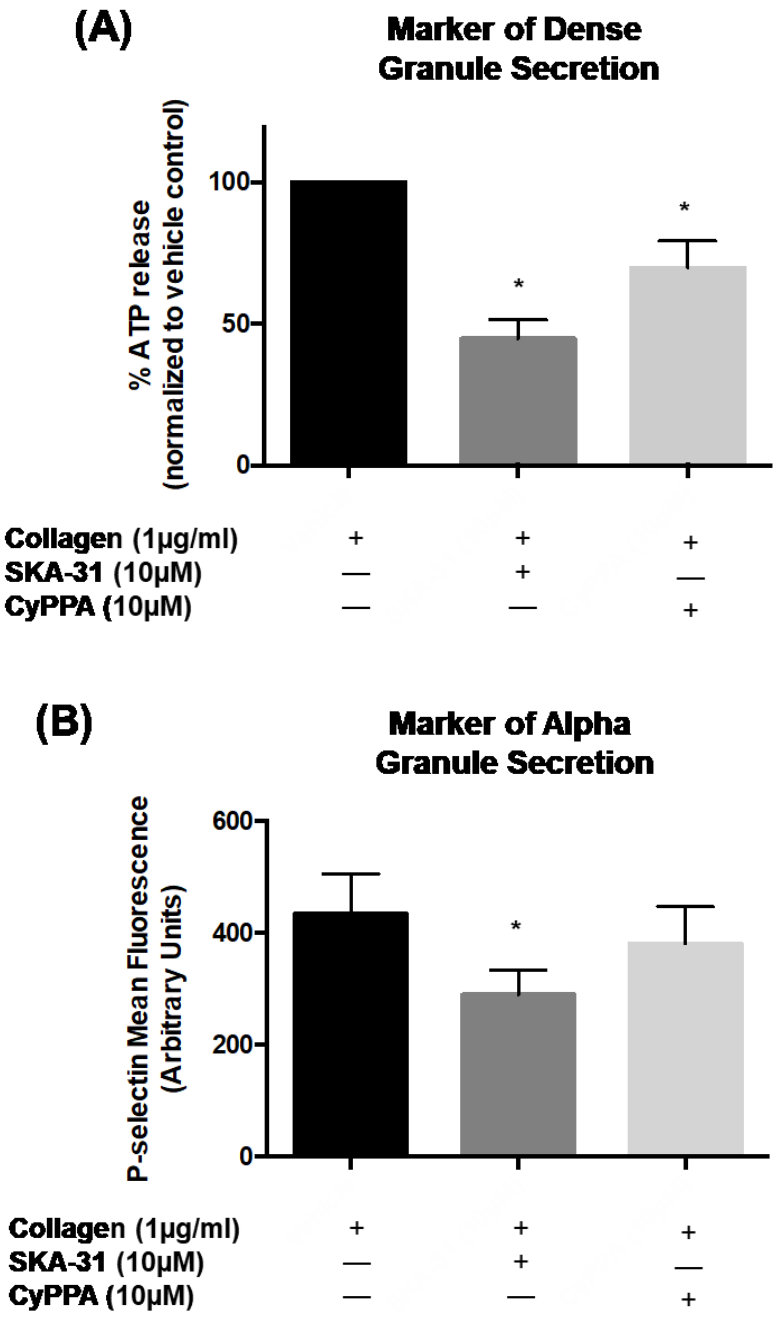

Figure 18. Investigation of dense and alpha granule secretion. (A) quantified data of dense granule secretion, as measured using ATP chemiluminescence. (B) Alpha granule secretion, as measured using P-selectin antibodies and flow cytometry. Statistics: RM one-way ANOVA. N=5, both sets of experiments. *P<0.05 to vehicle control.

5. DISCUSSION

The primary responsibility of platelets is the maintenance of hemostasis, thereby stopping bleeding upon vascular injury. However, due to uncontrolled thrombus formation, the pathological extension of hemostasis – thrombosis – can be deadly. It is estimated that almost one half of all cardiovascular deaths in Canada are due to ischemic events such as ischemic stroke or myocardial infarction.[2] Statistics such as these make the growing understanding and knowledge of platelet biology, and the search for treatment options ever more pertinent.

Presently, most anti-platelet therapies are used for secondary prevention of thrombotic events. The mechanisms by which antiplatelet drugs inhibit aggregation include: inhibition of COX1 with acetylsalicylic acid, P2Y12 receptor binding with drugs such as ticagrelor and clopidogrel, and GPIIb/IIIa inhibitors such as eptifibatide. Although these treatments are well established, and fairly well tolerated, one of their major side effects is the risk of bleeding which can in itself be life threatening. The risk of bleeding occurs as a result of inhibition of platelet function, thus not only targeting and inhibiting thrombosis but also limiting any hemostatic function. As such, the ideal drug candidate would be a drug that could inhibit or limit thrombus formation with minimal inhibition of hemostasis, thus maintaining the ability to stop instances of bleeding.

Current anti-platelet therapies target different aspects of the platelet system, such as the enzyme and receptors described above, however there are currently no drugs that target ion channels in platelets. Ion channels contribute to platelet function by regulating the resting membrane potential, or contributing to regulation of calcium signalling, both of which contribute to platelet aggregation and function. Thus, the identification of calcium-activated potassium channels in platelets offers the opportunity for new insight into how platelets function and

possibly provides a new anti-platelet drug target. Since not much is known about the function of these channels within platelets, this study aimed to identify and characterize the effects of K_{Ca} channels on platelet activation and aggregation.

Although little is known of the role of K_{Ca} channels in platelets, lessons may be learned from their role in regulating endothelial function. The role of calcium-activated potassium channels in endothelial cells is well established, alongside their mechanism of action. In endothelial cells, activation of SK_{Ca} or IK_{Ca} channels caused as a result of calcium release from intracellular stores, will result in K^+ efflux and subsequent membrane hyperpolarization.[133] This hyperpolarization of the endothelial cell membrane results in Ca^{2+} entry via TRPC channels, thereby stimulating eNOS to generate more NO, thus relaxing the adjacent vascular smooth muscle.[159] This Ca^{2+} entry will simultaneously continue to feed forward this mechanism by continuing the activation of the K_{Ca} channels.

Based on the role and mechanism of action of K_{Ca} channels in endothelial cells, the hypothesis for this study was that pharmacologically activating either IK_{Ca} or SK_{Ca} channels in platelets would result in K^+ efflux, and thus membrane hyperpolarization, followed by Ca^{2+} entry, potentially in a localized manner, thereby stimulating NO generation by eNOS and resulting in inhibition of platelet aggregation in an autocrine manner. Activation of K_{Ca} channels would thus potentially provide a very novel drug target that would enhance an intrinsic negative feedback pathway within platelets. The papers by Fine et al., and Mahaut-Smith set the groundwork for this study by identifying the presence of K_{Ca} channels in platelets.[87, 115] Both papers provide evidence, although not definitive, suggesting that the K_{Ca} subtype present is likely IK_{Ca} not SK_{Ca}, that they are present at a low copy number (5-7 channels per platelet), and that they may be involved in regulating membrane potential.[87, 115] However, these papers simply

identify the presence of K_{Ca} channels in platelets without further characterization of their role in platelet function, which is the reason for this study.

The first objective of this study was to confirm which subtypes of K_{Ca} channels are present in platelets and their localization within the platelet. This was performed using confocal microscopy of platelets stained for IK_{Ca} and SK_{Ca} channels with channel specific antibodies, and blocking peptides, Figure 7. The results demonstrate clear presence of IK_{Ca} channels in every donor, but SK_{Ca} channels were only detected in two out of five individuals. When used, the blocking peptides successfully inhibited fluorescence, confirming presence of the channels. These findings suggest that the IK_{Ca} channels may be the dominant K_{Ca} channel present in platelets, which is in agreement with results presented by both Fine et al., and Mahaut-Smith's findings.[87, 115] Subsequent microscopy used platelets stained for the antibodies and co-stained for F-actin (Figure 8. **Representative confocal microscopy of distribution of KCa channels in platelets.**), to determine localization within the platelet. The overlap of green and red fluorescence suggests localization of the IK_{Ca} channels to the plasma membrane. Unfortunately, either due to the presence of phalloidin, or simply due to the individuals used, SK_{Ca} channels were not detected in this set of microscopy.

The next objective was to study the effects of pharmacologically activating IK_{Ca} and SK_{Ca} channels on platelet aggregation. As initially hypothesized, the activation of IK_{Ca} and SK_{Ca} channels by SKA-31 or CyPPA, respectively, demonstrated a concentration dependent inhibition of platelet aggregation in response to collagen (Figure 9 and Figure 10). The 10μM concentration was subsequently chosen for both compounds, since it generated approximate half-maximal inhibition.

In vivo, upon injury platelets are exposed not only to collagen, but other components of the subendothelial matrix, and other pro-aggregatory factors such as thrombin, and ADP. Therefore, the inhibitory response of K_{Ca} channel activation using an alternate platelet agonist, thrombin, was investigated (Figure 11). The results with thrombin follow a similar trend to the collagen responses, although the platelet inhibition by either K_{Ca} channel activator is much less dramatic, and subsequently statistically non-significant, demonstrating no differences between groups. This is unsurprising, since the thrombin concentration response curve is very steep, due to the relative strength of thrombin as a platelet agonist.[160] For this same reason, thrombin proved quite challenging to titrate appropriately for each given donor. Even though the results are not statistically significant, a concentration response curve was not generated, and thus it is entirely possible that higher concentrations of SKA-31 or CyPPA, such as the 100μM used for collagen experiments, may inhibit platelet aggregation in response to thrombin.

The next set of results, Figure 12, were designed to help confirm the actions of SKA-31 and CyPPA on their targeted K_{Ca} channels, IK_{Ca} and SK_{Ca} respectively. This was accomplished by using the respective blockers of the channels in combination with the activators. Since the blockers demonstrate higher selectivity for their respective channels, with the K_d for TRAM-34 at 20nM and the K_d for apamin in the10-30pM range, the design of these experiments would help determine if the activators act through their targeted channel in platelets.[156, 157] Despite the K_d's for the inhibitors being so low, the concentrations chosen are in alignment with concentrations used in studies on endothelial cells.[149] The action of the IK_{Ca} activator, SKA-31, was successfully blocked by the IK_{Ca} channel blocker, TRAM-34, whereas apamin demonstrated no significant effect in reversing SKA-31 platelet aggregation inhibitory effects. This suggests SKA-31 is acting through IK_{Ca} channels but not SK_{Ca} channels, which as the

intended target channel confirms the actions of SKA-31. This is in contrast to the results obtained with CyPPA, where the inhibitory action of CyPPA on platelet aggregation were not reversed by any K_{Ca} channel inhibitors used. These results may indicate that CyPPA is having a non-specific effect, independent of IK_{Ca} or SK_{Ca} channel function, on platelets that manages to inhibit platelet aggregation. These findings would concur with findings in the study by Fine et al., using the fluorescent indicator DiSC3(5) to study platelets, demonstrated that platelets did not respond to apamin.[115] Similarly, in Mahaut-Smith's study on platelet K_{Ca} channels, the recorded conductance of channels was 30pS, the conductance displayed within the range for IK_{Ca} channels.[87] However, when studying the effects of TRAM-34 or apamin alone, Figure 13, apamin significantly inhibited platelet aggregation alone, and explains why it would be unable to reverse the effect of CyPPA. In effect both the activator and the blocker of SK_{Ca} channels inhibited platelet aggregation, which may suggest that either opening or closing the channel may have a significant enough effect on platelets to inhibit their function, which requires further investigation. Additionally, these results may help explain the results obtained by microscopy from this study, Figure 7 and Figure 8, where only two individuals were found to have any SK_{Ca} channels, but every individual demonstrated presence of the IK_{Ca} channel. Thereby suggesting that IK_{Ca} channels are the dominant K_{Ca} channel present in platelets, and the results with SK_{Ca} channels being somewhat inconclusive. Further exploration of the presence, absence and function of SK_{Ca} channels would need to be investigated.

Despite its incompletely understood function in platelets, CyPPA was still used throughout this study to further characterize its effects on platelets. Based on the inhibitor experiments, CyPPA and apamin both inhibited platelet aggregation, and therefore CyPPA may or may not function through SK_{Ca} channels, making its mechanism of action somewhat unclear.

Platelets were treated with the K_{Ca} channel inhibitors, TRAM-34 and apamin alone to determine if inhibiting the channels has any effect on platelets. The results in Figure 13 demonstrate that TRAM-34 has no effect on platelets alone, whereas apamin inhibited platelet aggregation, discussed briefly above. The intention of these experiments was to determine whether the channels are open or closed in platelets. The interpretation of these results suggests that under resting, non-aggregating conditions of platelets IK_{Ca} channels are inactive, since inhibition IK_{Ca} channels has no effect on platelet aggregation. This may suggest that the channels play a role in regulating platelet function by control of membrane potential. This is a unique finding, as it offers a possible target that inhibits platelet aggregation, but otherwise may not have a function. SKA-31 and CyPPA activate IK_{Ca} and SK_{Ca} channels by increasing their sensitivity to Ca^{2+}, by shifting the Ca^{2+}-activation curve towards lower concentrations of Ca^{2+}.[138] Since the channels are being activated pharmacologically in this study, the mechanism by which these channels may be activated under physiological conditions remains not fully understood, although the involvement of Ca^{2+} is clear.

Since *in vivo,* the flow of the blood is laminar, a novel model to study laminar flow was used, the Q-sense™ QCM.[153] These experiments more closely mimic a physiological model of blood flow and thus make them very relevant to further understanding the function and responses of platelets physiologically. Figure 14 demonstrates the results of frequency and dissipation measured by the QCM balance, which show a decrease in dissipation, without a significant change in frequency as a result of SKA-31, the IK_{Ca} channel activator, treatment when compared to vehicle control. A change in dissipation, without a change in frequency suggests inhibition of aggregation, without an effect on the overall mass of the adhered platelets. This was confirmed when visualizing the aggregates on the sensors, with F-actin stained

platelets, under the confocal microscope. The images in Figure 15 are displayed as maximum projections of Z-stacks taken of the aggregates on the sensors; maximum projections put the 3-dimensional component of the image, in this case the buildup of the aggregate, onto one plane so that it can be visualized in a 2-dimensional fashion, so what is seen is the entire aggregate at once. Figure 15 visually confirms the findings that SKA-31 treatment inhibited platelet aggregation.

One of the major hypotheses for this study, as based on endothelial cells, was that aggregation would be inhibited due to an increase in platelet NO generation. Since NO is one of the main endogenous inhibitors of platelet function, this could therefore provide a novel drug target, targeting the autocrine/endogenous negative feedback system of platelets. To investigate this aim, DAF-FM stained platelets were used and processed on the flow cytometer, after performing aggregation and labelling with the anti-CD42b-PE antibody, used as a platelet marker. The results in Figure 16 unexpectedly demonstrate that the activation of IK_{Ca} (and SK_{Ca}) channels results in the inhibition of platelet-generated NO and are more similar to the resting platelet control. This suggests that platelet aggregation is inhibited by IK_{Ca} or SK_{Ca} channel activation, independent of NO-signalling. Since both aggregation and NO generation are Ca^{2+}-dependent processes, it may indicate that Ca^{2+}-signalling is inhibited by activation of IK_{Ca} or SK_{Ca} channels.

Calcium is a key factor in signalling within many cell types, and this applies to platelets as well. Ca^{2+} is the driving factor for a lot of platelet function, including initiation of aggregation, spreading of pseudopodia as a result of initiated aggregation, NO generation via eNOS, and granule secretion. Since Ca^{2+}-signalling is involved in all these pathways, Ca^{2+}-signalling was studied in an attempt to explain the actions of the K_{Ca} channel activators. Figure 17 demonstrates

the inhibition of Ca^{2+} signalling in platelets undergoing aggregating conditions when incubated with SKA-31, the IK_{Ca} channel agonist, with CyPPA having a non-significant effect on calcium signalling compared to vehicle control. Since the majority of aggregation dependent processes are also calcium-dependent, this explains the inhibition of aggregation of platelets both in the aggregometer and under laminar flow conditions, as well as the inhibition of NO generation via eNOS, and the inhibition of both dense and alpha granule secretion, by SKA-31.

Another aspect of platelet machinery involved in aggregation, that is also Ca^{2+}-sensitive, is granule secretion. Therefore, dense and alpha granule secretion was further characterized here in response to K_{Ca} activation. Figure 18 demonstrates inhibition of ATP secretion from dense granules by both SKA-31 and CyPPA, and inhibition of P-selectin secretion from alpha granules by SKA-31. Since granule secretion occurs as a result of platelet activation in the aggregation pathway, and is Ca^{2+}-dependent, it may be anticipated that granule secretion would likewise be inhibited, which holds true for treatment with SKA-31. The inhibition of dense but not alpha granule secretion of CyPPA is unusual, but may suggest targeting of specific PKC's such as PKCα or PKCβ, or targeting of specific vesicle-SNAREs (VAMPs).

In studies on VAMPs in knockout mice in regards to granule secretion, it has been determined that about 50-60% of dense and alpha granule secretion is needed for thrombus formation, with a limited effect on bleeding time.[12] In effect, this suggests an inhibition of 30-40% of granule secretion for this to hold true. If this is indeed the case, this again makes a good argument for targeting IK_{Ca} channels in platelets, which are seen as inhibiting granule secretion in the 30-50% range (Figure 18), as a potential means to limiting the process of thrombosis with a minimal effect on hemostasis.

The first major study to identify the presence of K_{Ca} channels in platelets, utilized fluorescent indicator DiSC3(5) to measure hyperpolarization of the platelet membrane.[115] The results from this study lead to the conclusions that the type of K_{Ca} channel present in platelets may be involved in regulating membrane potential, and is likely to be IK_{Ca} subtype, since there was no response to SK_{Ca} inhibitor apamin. The next major study on K_{Ca} channels in platelets, utilized whole-cell patch clamping of platelets and similarly concluded that the channels may be involved in regulating membrane potential, and are inactive at rest but respond to Ca^{2+} signalling.[87] This study also concluded that the channels are likely IK_{Ca}, based on the conductance recorded of 30pS, and that platelets only contain a low number of the channel (5-7).[87] These previous studies lay the ground work for the findings in this study. When identifying channel subtypes, both IK_{Ca} and SK_{Ca} channels were detected, although the IK_{Ca} channels seem to be the dominant K_{Ca} channel in platelets, and were identified in all individuals. Additionally, the IK_{Ca} channels are clearly identifiable within the platelet plasma membrane. The use of IK_{Ca} channel blocker, TRAM-34 suggests that the channels are inactive at rest, which is in agreement with Mahaut-Smith's findings.[87] In the study by Fine et al., a lack of response to apamin was demonstrated, in contrast here apamin was able to inhibit platelet aggregation.[115] Perhaps suggesting that when SK_{Ca} channels are present, they contribute to the membrane potential, activation or blocking of which may result in a significant enough hyperpolarization or depolarization of the platelet membrane resulting in inhibition of platelet aggregation.

5.1 LIMITATIONS OF THIS STUDY

Limitations exist within any study, and thus this study is no exception. One of the most notable limitations is the use of exclusively healthy human volunteers, who donated their platelets, thereby limiting the scope of this study to healthy individuals. However, in order to determine the effectiveness of targeting platelet K_{Ca} channels as a therapy for cardiovascular disease, the use of an exclusively healthy population is not relevant.

A limitation in the experimental design is the use of only one channel activator, SKA-31 for IK_{Ca} channels and CyPPA for SK_{Ca} channels. Other, more potent activators exist for both channels, including SKA-111 and SKA-121 for IK_{Ca} channels and NS13001 for SK_{Ca} channels. Use of additional activators could help corroborate the presence or absence of SK_{Ca} channels, and may aide in delineation of CyPPA's possible mechanism of action.

We attempted to use as physiological a model as possible, which was the Q-sense™ QCM, which allows for laminar flow conditions. This is still not the same as using an *in vivo* model, which is only possible with the use of animal models, such as mice or rats. Although animal models can provide lots of information, they present difficulty when translating to human physiology.

A major limitation is the lack of whole-cell patch clamping which could have provided clearer results for the Ca^{2+}-signalling experiments in platelets with K_{Ca} activation, as well as measuring membrane potential changes in response to the activators. Attempts were made to patch clamp on platelets, but due to their small size this proved rather challenging. Further attempts were made using Meg-01 cells, a megakaryoblastic cell line, as a surrogate for platelets, which also provided limited success due to their inherent lack of adherence over longer periods of time, as well as their tendency to die rather quickly after removal from the incubator.

6. CONCLUDING REMARKS

In endothelial cells, activation of IK_{Ca} or SK_{Ca} channels results in potassium efflux and subsequent hyperpolarization of the cell membrane. This hyperpolarization results in calcium influx via TRPC channels, which feeds forward to stimulate eNOS to generate more NO. Since platelets have the ability to generate NO and the presence of K_{Ca} channels identified, this study aimed to investigate the effect of the IK_{Ca} and SK_{Ca} channel activators on platelet function and platelet-NO generation. The main hypothesis was that platelet aggregation would be inhibited upon K_{Ca} channel activation, due to potentially an increase in platelet-NO generation. This study demonstrates that:

1. IK_{Ca} channels are the dominant K_{Ca} channel within platelets, as demonstrated using confocal microscopy.
2. Pharmacological activation of IK_{Ca} channels or use of CyPPA on platelets inhibits aggregation to collagen.
3. The SK_{Ca} channel activator, CyPPA, may not function through SK_{Ca} channels in platelets, and requires further investigation.
4. Inhibition of platelet IK_{Ca} and possibly SK_{Ca} channels has no effect on aggregation, suggesting that these channels are not open under resting conditions.
5. Under laminar flow conditions, IK_{Ca} activation was able to inhibit aggregation, but not adhesion of platelets.
6. Unexpectedly, NO generation is inhibited by activation of IK_{Ca} channels and with use of CyPPA
7. Calcium signalling decreases significantly in platelets treated with the IK_{Ca} channel activator, SKA-31.

8. SKA-31 and CyPPA both inhibit dense granule secretion, while only SKA-31 treatment inhibits alpha granule secretion

7. FUTURE DIRECTIONS

The results of this study, did demonstrate inhibition of aggregation (Figure 9 and Figure 10, but independently of NO-generation (Figure 16). Due to this unexpected result, this study further characterized the effects of channel activators SKA-31 (IK_{Ca}) and CyPPA (SK_{Ca}) on platelets, demonstrating inhibition of several aspects of platelet aggregatory machinery as a result of inhibited calcium signalling by activation of IK_{Ca} channels.

Although Mahaut-Smith's original study on K_{Ca} channels involved patch clamping, he did not use the K_{Ca} channel activators, which would be relevant to study in the future.[87] However, despite the difficulties presented by patch-clamping of platelets and Meg-01s, the fact remains that patch-clamping could provide better insight into the inhibition of calcium signalling resulting from IK_{Ca} channel activation. Since the calcium signalling results demonstrate that activation of IK_{Ca} channels inhibits calcium signalling, Figure 17, but the source and regulation of this downstream calcium is unknown. Additionally, patch-clamping would provide concrete data on changes in the membrane potential of platelets in response to the K_{Ca} channel activators. An attempt to measure membrane potential at different stages in aggregation, similar to the calcium signalling experiments was made, using dye $DiBAC_4(3)$ (1μM), but the results were inconclusive due to inconsistency in the measurements, Figure 19.

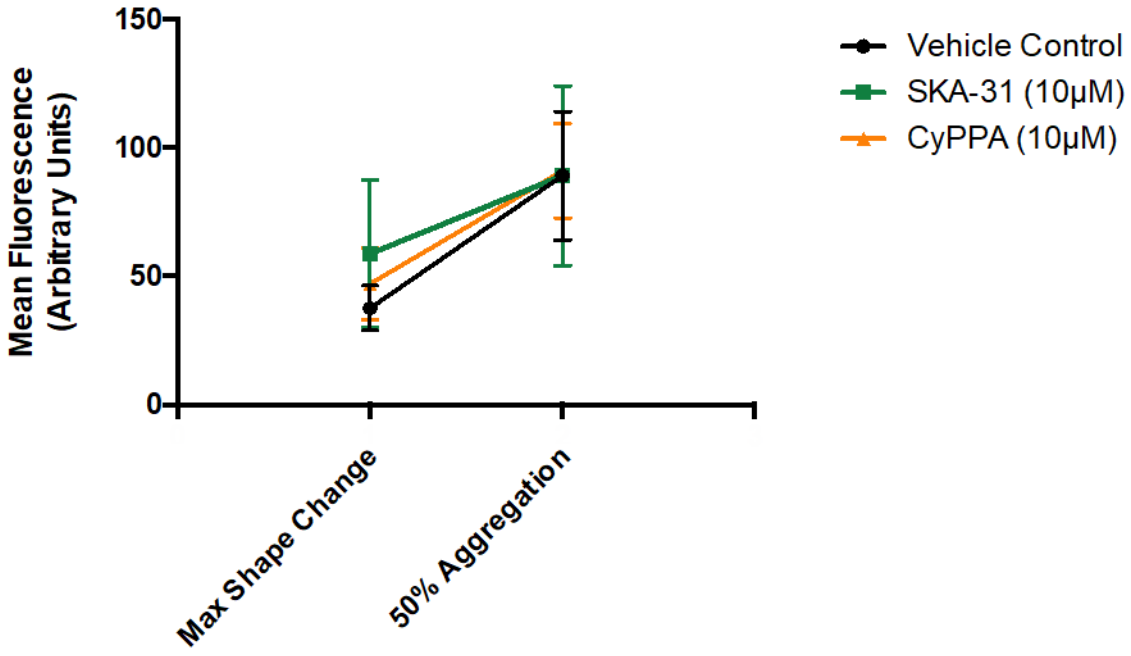

Figure 19. Summary Results of Attempted Membrane Potential Measurements.

More exciting next steps include the possibility of using an *in vivo* animal model, such as mice to study the effects of SKA-31 and CyPPA on thrombus formation, bleeding time, as well as vasodilation. Although this has previously been attempted to study vascular relaxation in response to the IK_{Ca} and SK_{Ca} channel activator by Dr. Frances Plane, little progress was made due to issues with the solubility of the DMSO vehicle. However, salts of the K_{Ca} channel activators have been created, in the hopes for an *in vivo* study, which hopefully rectify this issue. An *in vivo* model could also provide interesting insight on activation of K_{Ca} channels both in endothelial cells as well as platelets, and any synergistic effects that may result.

Early on in this study, the compound CyPPA, the SK_{Ca} channel activator was thought to be acting non-specifically, when used in combination with the SK_{Ca} channel blocker, apamin, Figure 12. However, as demonstrated in Figure 13, apamin was able to inhibit platelet aggregation, therefore CyPPA may well be acting through SK_{Ca} channels, but requires further confirmation. Additionally, SK_{Ca} channels were only detected in some individuals in the microscopy experiments, which could potentially contribute to an explanation of CyPPA's actions. It would be incredibly relevant to repeat several experiments, to determine the effects of SK_{Ca} channel activation in populations where the channel is present and those where it is absent.

The major question that remains is whether IK_{Ca} and SK_{Ca} channels in platelets are simply vestigial remnants from the parent megakaryocyte. The use of channel blockers in platelets demonstrated that IK_{Ca} channels are likely to be inactive at rest, whereas SK_{Ca} channels may exist in combination, Figure 13. Activation of the channels, by sensitizing their response to Ca^{2+} inhibited aggregation, by inhibiting intracellular Ca^{2+}. However, further elucidation of K_{Ca} channel function within megakaryocytes may further elucidate their purpose within platelets.

Since platelet NO-generation is inhibited by K_{Ca} channel activation, additional experiments may want to investigate the possibility of synergy, when used in combination with NO donors. In this same vein of studies, K_{Ca} channel activators in combination with other platelet antagonists, such as PGI_2, acetylsalicylic acid and P2Y12 antagonist would also be interesting to study, in order to allow for comparison but also investigate the possibility of any additive or synergistic effects.

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