University of Alberta

Manipulation of the Angiogenic Balance by Pharmacological Inhibition of Platelet PKC Signalling.

by

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To my family, always supportive.

ABSTRACT

Blood platelets mediate wound healing by preventing bleeding and by supporting growth of new blood vessels. Blood vessel growth is supported in part by growth factors released from platelet α-granules. Both platelet aggregation and α-granule release are mediated by intracellular signaling pathways via distinct protein kinase C (PKC) isoforms. Recent evidence suggests that the conventional PKC isoforms α and β are respectively responsible for signaling α-granule release and aggregation.

We hypothesized that titration of platelets with conventional PKC (cPKC) inhibitors will have differential effects on aggregation and the release of angiogenic factors from α-granules. Our results indicate that careful titration of cPKC inhibitors preferentially prevents the release of the pro-angiogenic factor VEGF but not the anti-angiogenic factor TSP-1 from platelet α-granules. Also inhibition of VEGF release from α-granules may occur independent of platelet aggregation. Hence, we have been able to uncouple angiogenesis-promoter from aggregation-stimulator abilities of platelets.

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LIST OF ABBREVIATIONS

Ca^{2+} - calcium

b-FGF - Basic fibroblast growth factor (FGF2)

BSA - Bovine serum albumin

cAMP - Cyclic adenosine monophosphate

cGMP - Cyclic guanosine monophosphate

COX - Cyclooxygenase

DAG - Diacylglycerol

DMSO - Dimethyl sulfoxide

FGF- Fibroblast growth factor

FGFR - Fibroblast growth factor receptor

GTP - Guanosine triphosphate

GPCR - G-protein coupled receptor

IP_3 - Inositol triphosphate

NO - Nitric oxide

PAR - Protease-activated receptor

PDGF - Platelet-derived growth factor

PDGFR - Platelet-derived growth factor receptor

PGI_2 - Prostaglandin I2

PLC - Phospholipase C

PKC – Protein Kinase C

PRP - Platelet-rich plasma

RP – Resting Platelets

SNAP - S-nitroso-N-acetylpenicillamine

TXA_2 - Thromboxane A

VEGF Vascular endothelial growth factor

VEGFR Vascular endothelial growth factor receptor

vWF - von Willebrand faktor

1. INTRODUCTION

1.1 Platelet Biology

Platelets are small subcellular fragments (2–5 μm diameter), derived from the cytoplasm and membranes of megakaryocytes, that circulate in the blood for 7–10 days at a concentration of 150–400 × 10^9 per liter. Platelets don't possess a nucleus; however, they contain other organelles such as large number of storage granules which are classified as α-granules, lysosomes, and dense bodies. α-granules (Figure 1) contain proteins involved in coagulation and cell adhesion, as well as growth factors, whereas dense granules are filled with small molecules such as ADP and serotonin. The platelet cytoskeleton forms a strong ring structure in the plane of the disc that maintains its shape. The plasma membrane features a large number of invaginations called the open canalicular system that increases the surface area and/or provides storage capacity for membrane components (White, 2007). Under physiological conditions, platelets circulate in a nonadherent "resting" state in the periphery of the bloodstream adjacent to the endothelium (Figure 1). Their activation is inhibited by nitric oxide (NO) and prostacyclin derived from endothelial cells. The key function of platelets is to prevent blood loss after injury by forming hemostatic plugs (Rex, 2007), thereby maintaining hemostasis.

When platelets participate in the haemostatic process a coordinated sequence of responses occur that include: 1) Adhesion: platelets in the circulation start adhering to exposed adhesive proteins or platelet agonists present in the basement membrane of the endothelium, such as collagen, vitronectin and von Willebrand factor (vWF). 2) Shape change: a rapid process in which the platelets change form from discs to spheres with extended pseudopodia. This highly dynamic process involves phosphorylation of the myosin light chain that enables the myosin monomers to polymerize and acquire a high affinity for actin, forming actin-myosin complex that causes the contraction of the cytoskeleton (Smyth,

Whiteheart, Italiano, 2010). Next filopodia protrude from the platelet membrane and during the adhesion process the platelets become spread out while stress-like fibers coalesce internally (Figure 1). 3) Aggregation: platelets bind to each other or aggregate by the binding of fibrinogen and also vWF to the integrin family of receptors (primarily α_{IIb}/β_3) forming platelet-platelet interactions (Figure 1). 4) Secretion: the retracted filaments center the cytoplasmic granules (α-granules and dense granules) within the platelet. Fusion of the granule membranes with the invaginations of the platelet membrane occurs next and then the granules release their contents to the exterior (Jennings, 2009; White, 2007; Li et al., 2010).

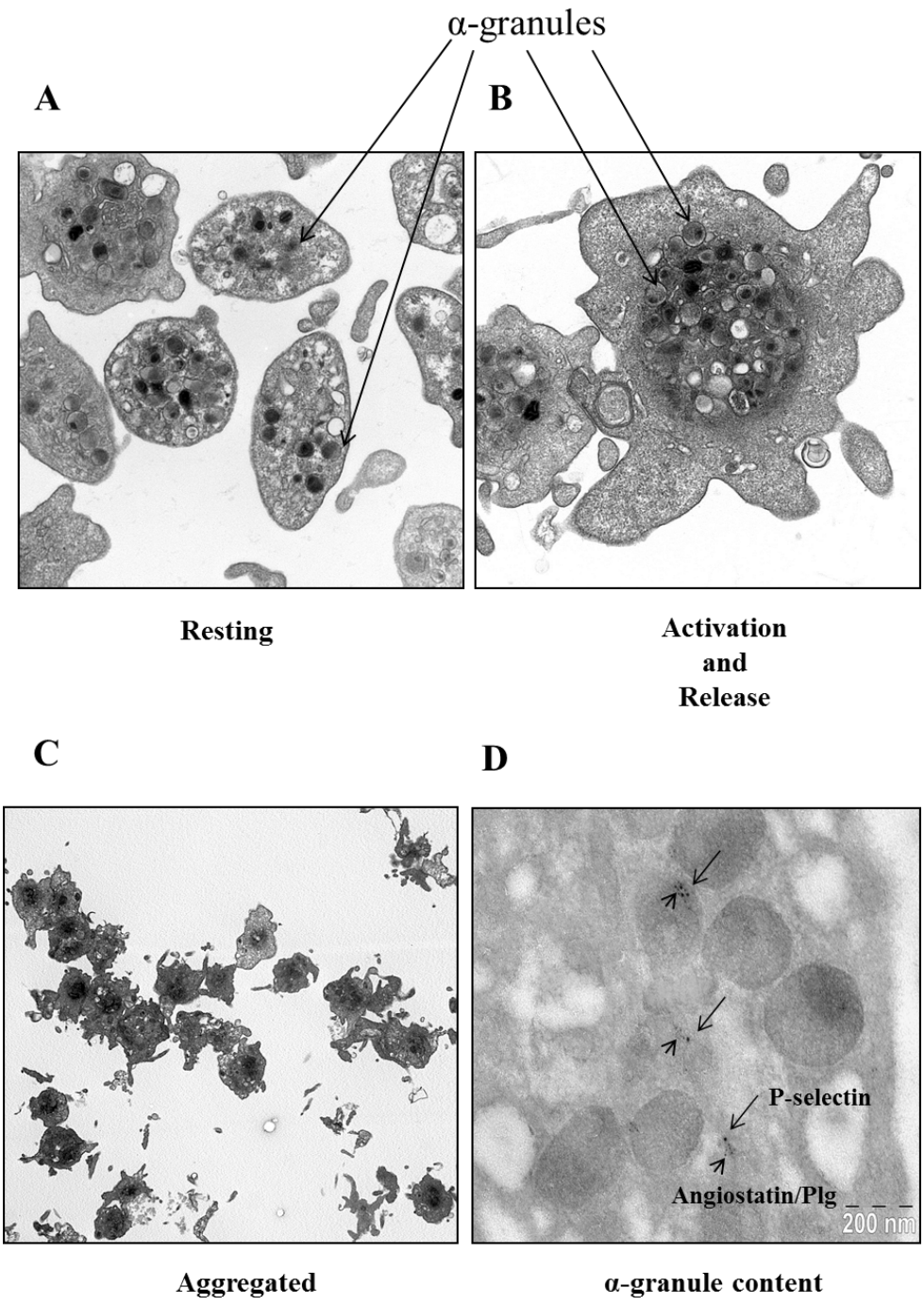


Figure 1: Electron microscopy of (A) resting platelets with discoid shape. (B) Activated platelets undergoing shape change and extending pseudopodia. (C) Aggregated platelets forming platelet-platelet interactions. (D) High-power dual immunogold EM of platelet α-granules and some of their contents including the receptor P-selectin, angiogenesis inhibitor angiostatin and its parent molecule plasminogen (Plg).

1.1.1 Platelet Agonists and Receptors

The physiological agonists for platelet signaling reported to date are: thrombin, ADP, epinephrine, serotonin, collagen, matrix metalloproteinases and arachidonic acid (Smyth, Whiteheart, Italiano, 2010; Öberg, 2009; Brass et al., 2007; Goggs and Poole, 2012; Santos-Martínez et al., 2008). Based on the receptors that the agonists activate, the agonists can be classified into two main classes: a) agonists that activate G- Protein Coupled Receptors (GPCRs) such as ADP, epinephrine, thrombin, TXA_2 and serotonin and b) agonists that activate non- G- protein coupled receptors such as collagen, vWF and fibrinogen (Holmsen and Weiss, 1979; Charo et al., 1977; Goggs and Poole, 2012; Smyth et al., 2009b; White, 2007; Li et al., 2010). Arachidonic acid and matrix metalloproteinase-2 released from the platelet cytosol, help as secondary signaling molecules to mediate and amplify aggregation by acting in an autocrine and paracrine manner, recruiting other platelets to the aggregate (Radziwon-Balicka et al., 2012).

The GP IIb/IIIa receptor (integrin α_{IIb}/β_3) is the most abundant receptor on the platelet surface, with 50,000 to 80,000 copies. The peptide sequence RGD (Arg-Gly-Asp) of adhesive proteins such as fibrinogen and vWF binds to the high-affinity state of GPIIb/IIIa (Nieswandt and Offermanns, 2004). The importance of GPIIb/IIIa is reflected by the severe bleeding phenotype of GPIIIa-deleted mice and patients with Glanzmann thrombastenia which have a severe bleeding tendency due to mutations in the genes encoding GPIIb or GPIIIa.

The GP Ib-IX-V complex is the major platelet receptor mediating interaction with vWF. This complex consists of leucine-rich repeat glycoproteins (Rivera et al., 2009). The role of platelet-specific glycoprotein GP Ib (a component of the GP Ib–V–IX complex) is to slow down and bind platelets, enabling cell signaling and activation. Its importance was discovered by studying patients who lack this protein (Bernard–Soulier syndrome) or in GP Ib-deficient mice, where a severe bleeding defect is observed (Boneu et al., 1984; Ware et al., 2000).

Thrombin is a serine protease that catalyzes the cleavage of fibrinogen into fibrin and of the platelet thrombin receptors (protease-activated receptors or PAR receptors). Thrombin cleaves the N-terminal domain of these PAR receptors to unmask the new N-termini, which further acts as tethered ligands to activate the receptor (Angiolillo et al., 2010). As a result thrombin activates both the coagulation pathway and platelets.

Collagen also is a potent agonist for platelet activation. The main platelet receptors for collagen are α2β1 and GP VI (Surin et al., 2008). GP VI has a short cytoplasmic tail that binds Fyn and Lyn Src kinases. GP VI is also constitutively complexed with FcR γ-chain dimer, which bears an immunoreceptor tyrosine-based activation motif (ITAM) acting as the signal-transducing subunit of the receptor (Rivera et al., 2009).

ADP is a weak agonist in comparison to collagen and thrombin. At least two ADP receptors are expressed in the platelet plasma membrane; both are G-protein coupled receptors. $P2Y_1$ couples to Gαq and contributes to Ca^{2+} mobilization, platelet shape change and rapidly reversible platelet aggregation. $P2Y_{12}$ couples to Gα12 and decreases cAMP, thus amplifying aggregation and platelet secretion (Patrono and Rocca, 2010; Offermanns et al., 1994).

1.1.2 Signaling Pathways of Platelet Activation

The signaling process that occurs during platelet activation can be classified into 3 stages: (1) the interaction of agonists with their respective platelet receptors and receptor-mediated early platelet activation signaling, (2) the intermediate common signaling events, and (3) integrin activation (inside-out signaling) and outside-in signaling (Rivera et al., 2009; Goggs and Poole, 2012).

The process of platelet activation begins when upon injury to a blood vessel; subendothelial molecules such as vWF and collagen are exposed to the circulation. These agonists interact with their respective platelet receptors. This initial interaction leads to a decrease in cyclic AMP (cAMP) and/or Ca^{2+}

mobilization (Davì and Patrono, 2007). The binding of agonists such as collagen or thrombin to their receptors on the surface of platelets, activates two membrane enzymes: phospholipase C and phospholipase A2. Activation of phospholipase A_2 leads to the release of free arachidonic acid which is converted by cyclooxygenase into prostaglandin endoperoxides to form aggregating thromboxane A_2 (TXA_2). TXA_2 amplifies the platelet adhesion signaling by binding to TPα and TPβ receptors, both coupled to G-proteins (Gq and $G_{12/13}$ families) (Rumbaut and Thiagarajan, 2010). TP receptors trigger phospholipase C pathway.

Activation of phospholipase C causes hydrolysis of membrane phospholipid phosphatidylinositol 4.5 bisphosphate (PIP_2), releasing diacylglycerol (DAG) and inositol triphosphate (IP_3). The IP_3 is involved in the movement of calcium into the platelet cytosol and stimulates phosphorylation of myosin light chains. The latter interacts with actin to facilitate the movement of the granules and the shape change of platelets. The DAG activates protein kinase C which, in turn, phosphorylates the downstream proteins that regulate the secretion of platelet α- and dense granules (Brass et al., 2007; Smyth et al., 2009b).

The centralization of α- and dense granules after platelet activation is caused by the platelet contractile apparatus. The next step after centralization is the fusion of granule membranes with the membranes of the intracellular canalicular system and external secretion of granule contents. The released granule molecules and the exposure of the P-selectin receptor help activate additional platelets. Although the initial signaling mechanisms of various platelet receptors differ, they ultimately converge into a final common pathway which is the promotion of the conformational change of GP IIb/IIIa receptors from a low affinity to a high affinity state for fibrinogen, a process known as inside-out signaling. Fibrinogen forms a bridge between individual platelets and facilitates hemostatic plug formation (Brass et al., 2007; Rex, 2007).

The change of conformation of GP IIb/IIIa initiates a series of intracellular signaling processes known as integrin outside-in signaling which amplifies platelet responses to GPCR agonists. GPCR and GP (glycoprotein) receptors are able to crosstalk with each other. GPCRs stimulate integrin activation and

regulate integrin outside-in signaling via $G\alpha_{13}$ subunit of GPCR. The $G\alpha_{13}$ subunit interacts with the β_3 cytoplasmic domain of GP IIb/IIIa. The β_3 cytoplasmic tail is required for outside-in signaling. More important, the GPCR/Gα13 and integrin outside-in signaling pathways coordinate with each other to dynamically regulate the signaling of RhoA, a member of the Ras family, which is critical for the processes of shape change, granule secretion and spreading by platelets (Li et al., 2010; Smyth et al., 2009a). In addition to their established role in hemostasis and thrombosis, platelets participate in inflammation, atherosclerosis, wound healing, antimicrobial host defense, malignancy and angiogenesis (Blair and Flaumenhaft, 2009; Jain et al., 2010a).

1.2 Beyond Hemostasis: The Platelet Contribution to Angiogenesis

Angiogenesis is the process of vascular growth by sprouting of capillaries from preexisting vessels. Physiological angiogenesis occurs in reproduction, development and wound repair. It is usually focal, such as following hemostatic reactions in a wound, and self-limited in time, taking days (ovulation), weeks (wound healing) or months (placentation) (Folkman, 2007). Angiogenesis is also involved in numerous pathological conditions such as atherosclerosis, macular degeneration, rheumatoid arthritis and cancer.

The role of angiogenesis in the development of solid tumors, its regulatory mechanisms and clinical implications in the management of cancer patients has been studied extensively since Dr. Judah Folkman's work in the early 1970s. Folkman hypothesized that "If a tumor could be stopped from growing its own blood supply, it would wither and die" (Folkman, 1971). Though his hypothesis was initially disregarded by most experts in the field, it is now widely accepted due to a great deal of subsequent research and the clinical success of angiogenesis inhibitors such as Avastin and Sorafenib in the treatment of cancer (Folkman, 2007).

For tumors to be able to grow they need to obtain nutrients and oxygen. Due to the limit of oxygen diffusion, for tumors to enlarge beyond 2 mm they need to develop new blood vessels to provide oxygen and nutrients to the tumor cells and also to remove waste products. Additionally angiogenesis is important for metastasis. Malignant cells may exit from a primary tumor into the blood circulation after the tumor becomes neovascularized (Makrilia et al., 2009). After arrival at distant organs, metastatic cells must again induce angiogenesis for a tumor to expand to a detectable size.

1.2.1 The Angiogenic Switch

Developing of new vessels occurs by a precise program of events. Signaling involved in angiogenic activation is relatively well known. The process begins with the signal for new vessel growth which is a consequence of injury or disease which often results in hypoxia. Hypoxia primarily up-regulates the expression of vascular endothelial growth factor (VEGF), a potent growth factor. The next step is endothelial activation, which occurs when growth factors such as VEGF or bFGF bind to receptors on the endothelial cell surface like the VEGF receptors (VEGFR). Once endothelial cells are activated, they attract and bind leukocytes, endothelial progenitor cells and blood platelets (Pinedo et al., 1998; Ramjaun and Hodivala-Dilke, 2009). Platelets release a multitude of pro- and anti-angiogenic factors which trigger local degradation of the basement membrane by enzymes like matrix metalloproteinase (MMPs) (Otrock et al., 2007). With the digestion of the surrounding matrix, the activated endothelial cells begin to migrate and proliferate in the direction of the stimulus (injured tissue or tumor). As vessels connect and form new vessel tubes these are stabilized by recruiting support cells (pericytes), such as fibroblasts and smooth muscle cells. At some point in these later stages, blood flow begins (Gerritsen, 2008).

The classical model of the regulation of angiogenesis is illustrated by a scale laden with anti-angiogenic molecules such as endostatin, platelet factor-4 (PF-4),

thrombospondin-1 (TSP-1), α_2-macroglobulin, plasminogen activator inhibitor-1 (PAI-1), and angiostatin (Radziwon-Balicka et al., 2012) on one side and pro-angiogenic molecules on the other, the so called “Angiogenic Switch” (Hanahan and Folkman, 1996). Induction of the angiogenic switch depends on how heavily that balance tips towards to pro-angiogenesis. Cancer cells begin to promote angiogenesis early in tumorigenesis (Bergers and Benjamin, 2003). This ‘angiogenic switch’ is characterized by oncogene-driven tumor expression of pro-angiogenic proteins, such as VEGF, basic fibroblast growth factor (bFGF), inter-leukin-8 (IL-8), placenta-like growth factor (PLGF), transforming growth-factor-β (TGF-β), platelet-derived growth factor (PDGF) and pleiotrophin. Tumor-associated hypoxic conditions also activate hypoxia-inducible factor-1α (HIF-1α), which promotes up-regulation of several angiogenic factors like VEGF, PLGF and Hepatocyte Growth Factor (HGF) (Kerbel and Folkman, 2002).

1.2.2 Tumor Angiogenesis and the Contribution of Platelets

In 1865, Armand Trousseau was the first to report an association between cancer and thrombosis in patients with gastric carcinomas (Varki, 2007). Later on Theodor Billroth in 1878 proposed that the spread of cancerous cells may be caused by tumor cell associated with thrombi. Since then evidence has shown that platelet aggregation by tumor cells plays a critical role in the pathology of metastasis (Jurasz et al., 2001a). In the late 1960s it was observed that decrease in platelet count (thrombocytopenia) in mice resulted in a 50% reduction in experimental metastasis (Gasic, 1968). Since then high platelet (thrombocytosis) counts found in patients with cancer assist in diagnosis and prediction of poor prognosis (Pinedo et al., 1998; Sierko and Wojtukiewicz, 2007). More recently it has been proven that most cancer patients have activated circulating platelets, without necessarily having a detectable wound or injury (Boneu et al., 1984; Bambace and Holmes, 2011) which has led to call tumors “wounds that never heal”.

The ability of tumor cells to activate platelets, tumor-cell induced platelet aggregation (TCIPA), provides a cancer cell a number of advantages for metastasis. Circulating platelet-cancer cell aggregates may protect cancer cells against shear forces and immune-mediated tumor cell clearance (Nierodzik and Karpatkin, 2007). In addition, platelet-tumor complexes induce downstream ischemic endothelial damage, which exposes adhesive molecules from the subendothelial matrix (fibronectin, von Willebrand factor). Both fibronectin and von Willebrand factor facilitates adhesion of cancer cells to both leukocytes and endothelial cells which aides in the extravasation of the tumour into the circulation (Jain et al., 2010a; Bambace and Holmes, 2011). Once the tumor cells have exited the circulation, factors derived from activated platelets such as VEGF, PDGF, bFGF, EGF, and MMPs (Jurasz et al., 2003a) promote angiogenesis, in that way enabling growth at the metastatic site (Gupta and Massagué, 2004).

Several studies have shown that serum concentrations of VEGF in cancer patients are link with platelet counts (Verheul et al., 1997; Salgado et al., 1999), and that cancer patients have elevated serum and platelet VEGF levels (Salven et al., 1999; Caine et al., 2004). VEGF content inside platelets increases during tumor progression (Salgado et al., 2001), and there is a shift in the platelet VEGF to TSP-1 balance favouring VEGF in cancer patients (Gonzalez et al., 2004). Studies using platelets of tumor-bearing mice have demonstrated that these platelets have higher intracellular levels of VEGF, bFGF, PDGF, and even TSP-1 and PF-4 during early tumor growth (Cervi et al., 2008; Klement et al., 2009; Zaslavsky et al., 2010; Radziwon-Balicka et al., 2012).

Recent proteomic analysis of platelets using mass spectrometry and two-dimensional electrophoresis, has reported over 300 proteins released during aggregation (Coppinger et al., 2004). This massive group of proteins includes cytokines, adhesive proteins, chemokines, coagulation factors and a wide variety of angiogenesis regulatory proteins (Table 1). Most of these angiogenesis regulatory proteins are stored in α-granules (Brill et al., 2004; Blair and Flaumenhaft, 2009). Under physiologic conditions, platelets release pro-angiogenic proteins from platelet α-granules to stimulate wound healing. These

pro-angiogenic proteins, include vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and matrix metalloproteinases (MMPs). These pro-angiogenic proteins are counter balanced by the release of angiogenic inhibitors from platelet α-granules, including thrombospondin-1 (TSP-1), endostatin, transforming growth factor-β1 (TGF-β1), platelet factor 4 (PF4), and tissue inhibitor of matrix metalloproteinases (TIMPs). These platelet-associated angiogenesis inhibitors are thought to stop uncontrolled blood vessel growth in later stages of healing in non-malignant wounds (Pietramaggiori et al., 2008; Browder et al., 2000; Daly, 2003; Radziwon-Balicka et al., 2012).

The general assumption in platelet biology has been that these various angiogenesis regulators are packaged at random in their granules. Thus, any given α-granule might contain some or all α-granule proteins. Also it was initially assumed that all these proteins were secreted together in an uncontrolled fashion (White and Rompietti, 2007). However, this assumption does not fit well with the model of the angiogenic switch because if platelets contain both pro- and anti-angiogenic factors, and if all are released at the same time they could potentially cancel out each other's effects (Italiano and Battinelli, 2009).

Ma and colleagues first discovered the selective release of platelet angiogenic proteins, by demonstrating that activation of proteinase-activated receptors (PARs), in particular PAR-1 and PAR-4 induce release of VEGF and endostatin from platelets in a differential manner. A PAR-1 stimulus induced VEGF release and suppressed endostatin release, while a PAR-4 stimulus induced endostatin release and suppressed VEGF release (Ma et al., 2005). These results were confirmed by Italiano and colleagues who demonstrated that angiogenesis regulatory proteins are in fact segregated among distinct sets of α-granules in platelets (Italiano et al., 2008). In particular, the major pro-angiogenic regulatory protein VEGF is stored in one set of α-granules, whereas the anti-angiogenic regulatory protein endostatin is packaged into another set of α-granules. Double immunofluorescence labeling of VEGF and endostatin or thrombospondin-1 and bFGF, was used to confirm the segregation of stimulators and inhibitors of

angiogenesis into separate α-granules in human platelets (Italiano et al., 2008). Similar granule heterogeneity was reported by Seghal and Storrie, who demonstrated that von Willebrand factor and fibrinogen are as well located in separate α-granules in human platelets (Sehgal and Storrie, 2007). More recently it has been demonstrated that the ADP receptors, P_2Y_1 and P_2Y_{12} are also involved in the regulation of angiogenic protein exocytosis although this pathway seems to lead to less release of VEGF than PAR-mediated activation (Battinelli et al., 2011). This VEGF release can be abolished by selectively inhibiting the P_2Y_{12} receptor (Bambace and Holmes, 2011).

The source of the platelet-derived angiogenesis proteins remain under active investigation in both physiological and pathological conditions. Recently, it has been shown that circulating platelets can sequester angiogenesis regulatory proteins (such as VEGF, PF4 and TSP-1) in the presence of a tumour mass as seen in mouse tumour angiogenesis models. Increased levels of these factors are detectable in platelets, but not in plasma or serum, when dormant tumours are microscopic (<1 mm in size) (Klement et al., 2009).

Beside angiogenesis regulator uptake from the circulation, Zaslavsky et al. have recently demonstrated that TSP- 1, which is megakaryocyte derived, is up-regulated in the platelets of tumor-bearing mice and is a critical negative regulator during the early stages of tumor angiogenesis (Zaslavsky et al., 2010). Besides the megakaryocyte-derived and circulation-derived angiogenesis regulating proteins that are found in platelet α-granules, platelets also constitutively generate the anti-angiogenic regulator angiostatin on their membranes (Jurasz et al., 2006). Some of the platelet-generated angiostatin is shed into the circulation and some of it is taken up and stored in α-granules for release.

Table 1: Platelet derived molecules with known role in angiogenesis

Platelet-derived angiogenesis stimulators	Platelet-derived angiogenesis inhibitors
Vascular endothelial growth factor (VEGF) *	Transforming growth factor (TGF-β) *
Platelet-derived growth factor (PDGF) *	Endostatin *
Basic fibroblast growth factor (bFGF) *	Angiostatin *
Epidermal growth factor (EGF) *	Platelet factor 4 (PF4) *
Hepatocyte growth factor (HGF) *	Plasminogen activator inhibitor-1 (PAI-1) *
Insulin-like growth factor 1 and 2 (IGF-1 and IGF-2) *	Phosphoglycerate kinase (PGK) *
Platelet-derived endothelial cell growth factor (PD-ECGF) *	Thrombospondin-1 (TSP-1) *
Angiopoietin-1 (ANGPT1)*	Tissue inhibitors of metalloproteinases (TIMPs) *
Matrix Metalloproteinases 2 and 9 (MMP-2 and MMP-9) *	
Lipoprotein A (LPA)	
Sphingosine-1-phosphate (S1P)	
Stromal Cell-Derived Factor (SDF-1; CXCL12)	

(*) found in α-granules

1.2.3 The mechanisms of platelet α-granule release

α-granule contents are released when the α-granule membrane fuses with surface-connected membranes of the Open Canalicular System (OCS) or the plasma membrane (Blair and Flaumenhaft, 2009). Membrane fusion involves the formation of a fusion pore that represents the initial site of mixing of two membranes. This fusion pore rapidly expands until, in many cases, the granule membrane is entirely incorporated into the surface-connected membrane (or several granules are fused to form a large vacuole). Soluble NSF Attachment Protein Receptors (SNAREs) represent the core of the fusion machinery. They are membrane-associated proteins that are oriented to the cytosol. SNAREs associated with granules are termed vesicular SNAREs (vSNAREs). Platelet vSNAREs include SNARE attachment protein (SNAP)-23, SNAP-25; Vesicle associated membrane protein (VAMP)-3 and VAMP-8 (Ren et al., 2007; Polgár et al., 2003a). SNAREs associated with OCS or plasma membrane are termed tSNAREs, which in platelets include Syntaxins 2, 4 and 7 (Zimmerman and Weyrich, 2008). The association of vSNAREs and tSNAREs generates the energy required for membrane fusion by forming a four helix coiled coil complex that guides the fusion of the membranes causing the contents of platelet granules to exocytose (Sutton et al., 1998).

The function of SNAREs in platelet granule secretion must be tightly regulated so as to prevent the indiscriminant release of α-granule cargo and the current evidence suggests that the regulation of the process is controlled by Protein Kinase C signaling (Figure 2). PKC phosphorylates the SNARE molecule SNAP-23 with kinetics that parallel or precede granule secretion (Polgár et al., 2003a). PKC phosphorylation of Syntaxin-4 regulates α-granule secretion, while the dense granule secretion is regulated by the phosphorylation of syntaxin-2 (Flaumenhaft, 2003; Chung et al., 2000; Houng et al., 2003; Graham et al., 2009).

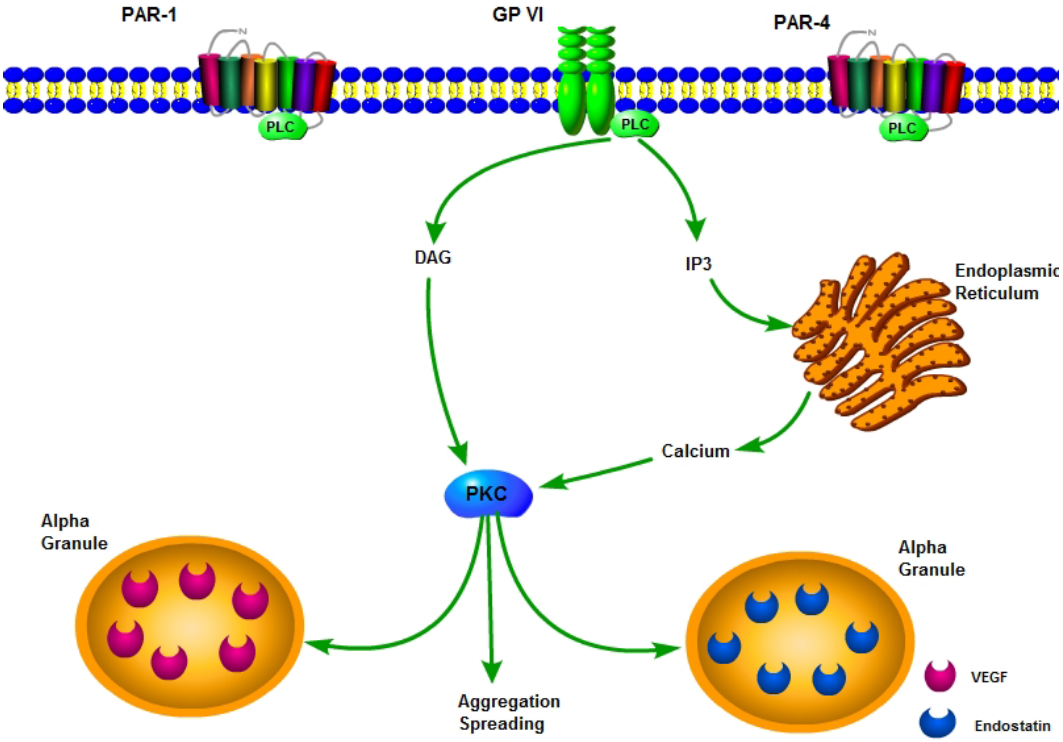


Figure 2: Adhesion to collagen, or stimulation by soluble agonists such as thrombin, activates numerous intracellular signaling molecules, especially PLC, resulting in a rise in [Ca^{2+}] and activation of PKC. PKC regulates many platelet responses to stimulation, such as granule secretion, aggregation and spreading.

1.3 Protein Kinase C

Protein Kinase C (EC 2.7.11.13) was discovered in 1977 by Nishizuka et al., who identified a kinase activated by treatment with calpain (a calcium-dependent neutral protease) in the rat brain. They observed that proteolysis of this enzyme generated two functional domains: one hydrophobic and one with complete catalytic activity. The latter was named PKM, as magnesium was the only cofactor required for activation. Because activation of the proenzyme required calcium and phospholipids, it was named PKC (Ohno and Nishizuka, 2002).

Protein Kinase C (PKC) is a family of enzymes that are involved in controlling the function of other proteins via phosphorylation which involves the transfer of a γ-phosphate group from ATP to the free hydroxyl groups of serine and threonine amino acid residues. The PKC family is comprised of 11 isozymes that are coded by 9 genes (Newton, 2001). This family of enzymes plays an important role in the signal transduction in the cell and participates in processes as diverse as cell proliferation, apoptosis, cell differentiation and regulation of the cell cycle. The PKC family proteins consist of a single polypeptide chain with a regulatory N-terminal region and a C-terminal catalytic region (Figure 3). The PKC structure consists of four conserved (C1–C4) and five variable regions (V1–V5). The C1 region contains an autoinhibitory pseudosubstrate sequence and contains the recognition site for phosphatidylserine, DAG, and phorbol ester. The C2 region of some PKC isoforms is rich in acidic residues and contains the binding site for Ca^{2+}. The C3 and C4 regions constitute the ATP- and substrate-binding lobes of the PKC molecule (Steinberg, 2008a). These regions are separated by a hinge sequence, which becomes very sensitive to proteolysis when the enzyme is attached to the plasma membrane. Proteolytic cleavage generates two fragments, corresponding to the regulatory region of approximately 30 kDa, and the catalytic region of about 50 kDa (Liu, 1996).

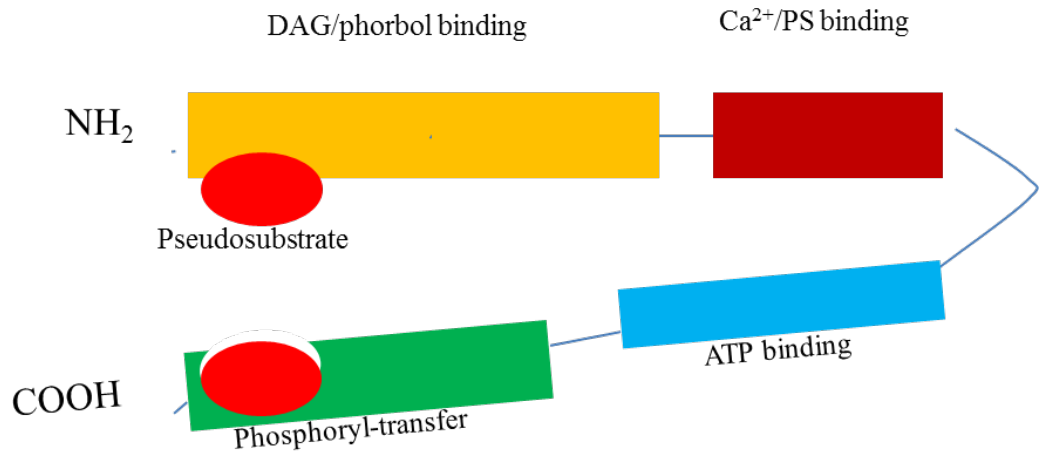


Figure 3: Structure of PKC. PKC has a C1 regulatory domain that contains a pseudosubstrate (PS) sequence followed by the regulatory domain C2 which contains the binding site for Ca^{2+}. The C3 contain the ATP-binding site while the C4 contains the catalytic domain. The PS domain has an autoinhibitory function in that it associates with the catalytic site on C4, thus preventing it from phosphorylating its substrates.

1.3.1 PKC isoforms

PKC isozymes can be grouped into three major classes taking into account their structure and the cofactors required for their activation: 1) conventional (α, βI, βII, γ) which are activated by both calcium and DAG that bind to C2 and C1 domains respectively of PKCs, 2) novel (δ, ε, η/L, θ) which are activated by only DAG and not calcium; and 3) atypical (μ , λ, ι, ζ) that are activated by lipid - derived second messengers that bind to the C1 domain and not by DAG or calcium (Nishizuka, 1995; Mellor and Parker, 1998; Toker, 1998; Newton, 2001). Most of the isoenzymes have an apparent molecular weight of 80 kDa, although it varies between 62 kDa and 97 kDa depending on the isoform. The optimum pH for PKC activity is between 7.5 and 8 and its isoelectric point is 5.6 (Liu, 1996).

1.3.2 PKC signaling and regulation

In its unstimulated state, most of the PKC resides in the cytosol. In this state, the pseudosubstrate sequence of the regulatory domain of PKC interacts with the catalytic domain and prevents access of the substrate to the catalytic site. Platelet PKC signalling is initiated, for example, upon binding of collagen to its membrane receptor resulting in activation of phospholipase C (PLC). The activated PLC hydrolyzes phosphatidylinositol-4, 5-bisphosphate (PIP_2) to produce DAG and inositol-1,4,5-trisphosphate (IP_3). The IP_3 causes the release of endogenous Ca^{2+} that binds to the cytosolic PKC and exposes the phospholipid-binding site. The binding of Ca^{2+} to PKC promotes its translocation to the plasma membrane via Receptor for Activated C - Kinases (RACKs), where it undergoes a conformational change. In its new form PKC interacts with DAG and Phosphatidylinositol- dependent Kinase 1 (PDK - 1), an upstream enzyme whose function is the transphosphorylation of the activation loop of the catalytic region (Steinberg, 2008b). The study of signal transduction pathways of the different isoforms of PKC is highly complex, because of the crosstalk and overlapping of

the different pathways that converge on the inside-out signaling of GP IIb/IIIa. Additionally the isoforms have high structural homology and functional overlap, making it difficult to determine the particular functions of each particular isoform (Steinberg, 2008b; Kiley et al., 1995). Enzyme inhibitors have been one of the main tools in the study of PKC-mediated signaling pathways. The major advantages of PKC inhibitors are that they allow high temporal and some spatial control and their effects can be observed in real time by live imaging, flow cytometry and other techniques (Eggert et al., 2006). PKC inhibitors also allow dose–response studies which are very informative. Their main limitation is possible non-specific effects, since PKC isoenzymes have highly conserved regions and share considerable sequence homology (Swannie and Kaye, 2002; Hu, 1996).

1.3.3 Inhibitors targeting PKC

In the case of PKC a number of inhibitory molecules have been described, with varying degrees of specificity (Hu, 1996). These inhibitors can be classified according to their mechanism of action: molecules that target the binding sites of phorbol esters or phospholipids (such as sphingosine or calphostin C), molecules that target the binding site of the pseudosubstrate domain or inhibitors that target the ATP binding domain, among which are the H7, staurosporine, K252, the indolocarbazoles or the bisindolylmaleimides (Swannie and Kaye, 2002; Hu, 1996).

Among the more specific inhibitors is LY333531, a competitive inhibitor that targets the ATP domain specific of the PKCβ isoform. Also rottlerin has been long considered a specific inhibitor of PKCδ. However studies have reported non-PKC specific effects of LY333531 and rottlerin showing that they can inhibit other PKC isoforms and even other kinases such as MAP kinases, casein kinase II, PKA and calmodulin kinase III (Way et al., 2000; Spitaler and Cantrell, 2004). PKC class selective inhibitors like Gö 6976 and Ro 32-0432, which selectively

inhibit the conventional PKC isoforms, are commercially available and previously used in the study of platelet function (Gilio et al., 2010a; Pula et al., 2005a). Besides the use of small molecule inhibitors, other pharmacological alternatives that have been used to reduce the activity of different PKC isoforms are membrane permeable pseudosubstrate inhibitory peptides (Hofmann, 1997).

1.3.4 PKC and platelet function

Protein Kinase C (PKC) signaling has been shown to play a role in platelet processes like Ca^{2+} entry, α-granule secretion (Yoshioka et al., 2001), αIIbβ3 activation and outside-in signaling (Buensuceso et al., 2005). PKC is also involved in receptor desensitization, extrusion of intracellular Ca^{2+}, secretion and filopodia formation (Harper and Poole, 2010; Gilio et al., 2010b; Jardin et al., 2007). Recent studies have focused on identifying downstream effectors of PKC, including SNARE proteins and their chaperones as some of them are phosphorylated by PKC in platelets (Woronowicz et al., 2010; Jardin et al., 2007). The phosphorylation of Myristoylated alanine-rich C-kinase substrate (MARCKS) by PKC precedes platelet granule secretion, and PKC pseudosubstrates derived from MARCKS inhibit granule secretion (Elzagallaai et al., 2009). A proposed mechanism of PKC regulation by MARCKS is that unphosphorylated MARCKS binds tightly to PIP_2, a lipid involved in granule secretion, in membranes and protects it from degradation by phospholipase C (PLC). After phosphorylation by PKC, the affinity of MARCKS for PIP_2 decreases allowing for other PIP_2-binding proteins to bind PIP_2. Because PIP_2 mediates platelet granule secretion, exposure of PIP_2 after MARCKS phosphorylation may contribute to granule secretion (Flaumenhaft, 2003).

At least 7 PKC isoforms, (α, β, δ, θ, η, ζ, ε) are expressed in platelets (Heemskerk et al., 2011) and there is evidence suggesting that each isoform may play a different role in platelet function. Data supporting isoform specific function is based on the use of isoform-selective inhibitors and experiments with

platelets of PKC deficient mice (Murugappan et al., 2004; Chari et al., 2009c; Konopatskaya et al., 2009; Harper and Poole, 2010; Way et al., 2000). PKCα has been identified as an essential factor in positively regulating platelet aggregation and both α-granule and dense-granule secretion (Wang, 2002; Xu et al., 2008; Yoshioka et al., 2001; Gilio et al., 2010b; Konopatskaya et al., 2009). PKCα null mice are unable to release their α-granule contents upon aggregation (Konopatskaya et al., 2009). These PKCα null mice also have a defect in platelet aggregation and thrombus formation because they also lack δ-granules and the ADP normally stored within them to mediate aggregation. Activation of platelets from PKCα null mice by exogenous ADP overcomes the aggregation defect but not the diminished α-granule release suggesting PKCα is very important to platelet granular secretion.

Studies using pharmacological inhibitors of PKCβ such as p99, a cell-permeable pseudosubstrate peptide inhibitor and by bisindolylmaleimide I, have shown that PKCβ is activated downstream of PARs and GPVI receptors and negatively regulates thrombin- induced calcium entry (Buensuceso et al., 2005). Also PKCβ has been found to immunoprecipitate with platelet integrin αIIb/β3, while PKCβ null mice display impaired spreading on fibrinogen (Buensuceso et al., 2005). Platelets from knockout mice for PKCθ and PKC δ have been shown to be activated by PARs and GPVI receptor stimulation but remain in an inactive state when stimulated by ADP (Nagy et al., 2009; Chari et al., 2009c). Both PKC θ and PKC δ positively regulate dense granule secretion, α-granule secretion and TXA_2 generation (Nagy et al., 2009). However, unlike PKC θ that positively regulates GPVI-mediated platelet functional responses, PKC δ negatively regulates GPVI- mediated platelet functional responses (Murugappan et al., 2004; Chari et al., 2009a). Nonetheless, the role of the novel PKC isoforms, PKCδ and PKCθ, is less straightforward, since there are studies that argue against these roles (Hall et al., 2008; Pula et al., 2006; Gilio et al., 2010a). Recent studies suggest that ADP activates PKCη via P2Y1 receptor and PKCη is subsequently dephosphorylated by αIIbβ3 mediated outside- in signaling (Bynagari et al., 2009). Roles for the PKCε and ζ isoforms in platelets are still not well known.

2. HYPOTHESIS AND OBJECTIVES

2.1 RATIONALE

The platelet contribution to tumor angiogenesis raises an important question. Could targeting of platelet granule release inhibit angiogenesis? Despite the current knowledge of the signaling events that regulate platelet aggregation and granule secretion, many questions remain to be answered. The mechanism that regulates the differential release of separate platelet α-granules and the underlying signal transduction pathways involved still remain unresolved.

The different discoveries on the signaling pathways that regulate the selective release of angiogenesis regulators from platelet α-granules described within the Introduction promise the potential to manipulate platelet secretion of angiogenic regulators from platelets for therapeutic purposes. The challenge in developing therapeutic strategies that inhibit platelet function is to avoid the risk of bleeding. However, it has been previously reported in the literature that is possible to uncouple aggregation from secretion (Rink et al., 1983). Recent findings have shown that protection of tumor vessel integrity by platelets requires platelet secretion (ie. granule release), but not plug formation. (Ho-Tin-Noé et al., 2008). The findings suggest that inhibitors that target platelet granule release without disturbing the hemostatic capabilities of platelets may allow for a new viable strategy to inhibit tumour angiogenesis.

Manipulation of the granule release and plug formation functions of platelet biology is possible by targeting the different signaling molecules involved in each process. Prime platelet signalling proteins whose pharmacological inhibition could potentially uncouple α-granule release from hemostasis are the PKC isozymes. In platelets, PKCβ has been shown to be involved in the phosphorylation and activation of GPIIb/IIIa receptor (Buensuceso et al., 2005), while PKCα phosphorylates components of granule secretory machinery (Yoshioka et al., 2001; Konopatskaya et al., 2009). These findings suggest that

selective PKCα inhibition would be expected to exert a major inhibitory effect on the release of platelet-associated angiogenesis regulators from α-granules while sparing primary platelet functions such as aggregation (Konopatskaya and Poole, 2010a).

2.2 HYPOTHESES

1. Selective PKCα inhibition uncouples platelet α-granule release from aggregation.
2. Inhibition of platelet α-granule release impairs platelet-stimulated angiogenesis by inhibiting the release of platelet-associated angiogenesis regulators.

2.3 OBJECTIVES OF THE STUDY

1. Identity the PKC isoform(s) responsible for signaling the release of angiogenesis regulator containing platelet α-granules.
2. Evaluate the effect of selective PKC inhibitors on angiogenesis regulator release from platelet α-granules during aggregation.
3. Assess the angiogenesis promoting activity of PKC inhibited platelets on angiogenesis *in vitro*.

3. MATERIALS AND METHODS

3.1 Reagents

Gö 6976, Ro32-0432, cell-permeable myristoylated nonapetide myr-FARKGALRQ, PKC β -inhibitor (3-(1-(3- Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino- 1H-pyrrole-2,5-dione) and Rottlerin were obtained from Calbiochem (San Diego, CA, USA). Collagen, thrombin and CHRONO-LUME reagent were purchased from Chrono-log (Haverston, PA, USA). Bovine serum albumin and phorbol myristate acetate (PMA) were from Sigma Aldrich (St. Louis, MO, USA). Matrigel and PE- labeled anti-human-CD62P (P-selectin) IgG1 mAb from BD Biosciences (San Jose, CA, USA). Antibodies for PKCα and PKCβI and βII were obtained from Abcam. Anti-Rabbit Dy-Light 548 and Anti-Mouse Dy-Light 488 were obtained at Jackson ImmunoResearch Inc (West Grove, PA, USA).

3.2 Isolation of Human Platelets

Approval for the current study was obtained from the Human Research Ethics Board at the University of Alberta. Whole blood (40 ml) was drawn from healthy, consented human volunteers who had not taken any drugs known to affect platelet function including nonsteroidal anti-inflammatory drugs (NSAIDs) for at least 14 days. Prostacyclin-washed platelets were prepared according to a previously established protocol (Radomski and Moncada, 1983). Briefly, blood samples were mixed with 3.8% w/v trisodium citrate (9:1 ratio). Then 0.06 µg/ml of prostacyclin (PGI_2) was added to prevent platelet activation during the isolation (Vargas et al., 1982). Subsequently platelets were centrifuged at 200g for 20 min (Eppendorf 5810R centrifuge, Hamburg, Germany). The resulting platelet-rich plasma (PRP) was mixed with PGI_2 (0.3 µg/ml) and later centrifuged again at 900g for 20 min, and the pellet containing platelets was resuspended in Tyrode's

buffer. The number of platelets was counted using a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter Inc. Fullerton, CA, USA) and adjusted to 2.5 × 10^8 platelets/ml.

3.3 Measurement of Platelet Aggregation

Aliquots (0.5- 1 ml) of washed platelet suspensions (2.5 x 10^8 platelets/ml) were pre-incubated at 37°C for 2 min in the presence of one of the following conventional PKC (cPKC) inhibitors Gö 6976 (0-300nM), Ro 32-0432 (0-10 μM), or membrane permeable myristoylated pseudosubstrate peptide inhibitor myr-FARKGALRQ (10 min pre-incubation) (0-1μM); or in the presence or vehicle. Thereafter, platelet aggregation was induced by adding thrombin (0.3 U/ml), collagen (10 μg/ml) or A549 lung carcinoma cells (4 x 10^4 cells/ml). Changes in light transmittance were recorded using a Chronolog Dual Channel Lumi-aggregometer (Model 560, Chrono-Log, Haverston, PA, USA) and monitored by AGGRO-LINK software for up to 15 min. After aggregation, platelet pellets and releasates were separated using centrifugation (900g for 10 min) and stored at -80 ^oC for further analysis of angiogenesis regulator release. Alternatively, to determine the passive release of angiogenesis regulators from non-activated platelets, platelets were treated in the same manner except physiological saline was added as vehicle instead of platelet agonists collagen and thrombin, or A549 cells.

3.4 Measurement of dense granule secretion by human platelets

ATP secretion from platelet dense granules was determined by chemiluminescence using the Luciferin-Luciferase assay. Assays were performed using 450 μL of washed platelets and 50 μL of 0.2 nmol/L luciferin-luciferase reagent (Chrono-Lume, Chrono-Log, Haverston, PA, USA). The platelets were

treated with vehicle or conventional PKC (cPKC) inhibitors Gö 6976 (0-300nM), Ro 32-0432 (0-10 μM) and peptide inhibitor myr-FARKGALRQ (0-1μM) for 2-15 minutes and then stimulated with collagen (10 μg/ml) in a lumi-aggregometer at 37°C with stirring at 900 rpm, and the corresponding luminescence was measured. A standard curve of the luminescence of an ATP standard (Chrono-Log, Haverston, PA, USA) at different concentrations (0-3 nM) was used to calculate the ATP released. The data was normalized to the maximum secretion and presented as the percentage of ATP released by platelets incubated with vehicle (% control).

3.5 Measurement of P-selectin expression by flow cytometry

Platelet α-granule release can be determined by measuring P-selectin exposure on platelets (Berman et al., 1986; Jurasz et al., 2001b). For this purpose an aliquot of 10 μL of washed platelets was removed from the platelet aggregometer following activation by collagen (10 μg/ml) or thrombin (0.3 U/ml). Platelets were removed at 25% aggregation to prevent the formation of large aggregates that can clog the flow cytometer. Platelet samples (10 μl) were diluted with 80 μL phosphate-buffered saline (PBS) solution and 10 μL PE labeled anti-CD62P (12.5μg/mL) (P-selectin) antibody (BD Biosciences, San Jose, CA, USA) for evaluation of α-granule release. Samples were incubated in the dark for 5 min at room temperature and then diluted with 900 μL of PBS. The samples were passed through the laser beam of a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter Inc. Fullerton, CA, USA) at a flow rate of 1,000 platelets per second. Light scatter and fluorescence data were obtained with gain settings in the logarithmic mode. Phycoerythrin (PE) fluorescence was detected in FL2 with a 585/42 filter. 10,000 events were collected and the platelet population P-selectin mean fluorescence was measured. Platelet P-selectin expression was expressed as percent of collagen-(10 μg/ml)-aggregated control.

3.6 Angiogenic protein quantification

Platelet α-granules contain a large array of angiogenesis regulating proteins (Table 1). Vascular endothelial growth factor (VEGF) isoform 165 (Brill et al., 2004) and thrombospondin-1 were measured as prototypical platelet pro- and anti-angiogenic molecules (Zaslavsky et al., 2010). Both molecules have been widely studied in the context of platelet-stimulated angiogenesis (Zaslavsky et al., 2010; Gonzalez et al., 2004; Ma et al., 2005) due to their abundance and potency making them easily detectable and physiologically relevant. Moreover, both molecules are packaged in different subpopulations of α-granules (Italiano et al., 2008). In addition, basic fibroblast growth factor (bFGF), a pro-angiogenic molecule present in platelet α-granules, was also measured (Brill et al., 2004). $VEGF_{165}$, bFGF, and TSP-1 concentrations in collagen-stimulated platelet releasates were measured in triplicate using the Quantikine human ELISA assay (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions using 200 µl of platelet releasate. Baseline levels of angiogenic regulators were also measured from resting platelets to determine the passive release of $VEGF_{165}$, bFGF and TSP-1. This baseline concentration of released angiogenesis regulators was then subtracted from collagen-stimulated releasates. The data was then normalized by expressing the results as percent of maximal release by collagen-aggregated platelets incubated with vehicle (% control).

3.7 Cell culture

A549 lung carcinoma cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Aldrich, St. Louis, MO, USA) with essential amino acids, gentamycin (0.05 mg/ml), penicillin (0.06 mg/ml), streptomycin (0.01 mg/ml), and 10% fetal bovine serum (Sigma Aldrich, St. Louis, MO, USA). The cells were supplied with fresh medium and subcultured three times each week.

Cells were detached from the flasks using EDTA (7mM) in DMEM with 10% fetal bovine serum and gentle shaking. EDTA was then washed away with Tyrode's solution, and the cells were pelleted by centrifugation (250g for 10 min), washed (3 times) and resuspended in Tyrode's solution at a concentration of 10^7 cells/ml as monolayers in 250-ml culture flasks.

Human Microvascular Endothelial Cells derived from lung (HMVEC-L) were obtained from Lonza (Walkersville, MD, USA). HMVEC-L passages 8–12 were maintained in Clonetics EGM-2 MV medium containing, gentamicin (0.05 mg/mL), penicillin (0.06 mg/mL), streptomycin (0.01 mg/mL) and 10% FBS until an 80% confluent monolayer was formed. The cells were supplied with fresh medium every 2 days and passaged once they were confluent. The cells were detached from flasks using a trypsin-EDTA solution. Both cell lines were maintained at 37^oC in a humidified incubator containing 5% CO_2.

3.8 In vitro angiogenesis assay

The effects of platelet PKC-inhibited releasates on angiogenesis were determined in vitro using a Matrigel capillary formation assay. For Matrigel experiments washed-platelet releasates were needed to be devoid of PKC inhibitors so as not to directly inhibit PKCs in capillary forming endothelial cells. Hence, isolated human platelet rich plasma (PRP) was incubated for 10 minutes with conventional PKC inhibitors Gö 6976 (100nM), Ro 32-0432 (1 μM), or peptide inhibitor myr-FARKGALRQ (1 μM). Then platelets were pelleted at 900 g for 10 minutes and resuspended in Tyrode's buffer (10 ml). Platelets were washed in this manner 3x to remove free inhibitors from platelet suspensions prior to aggregation. Washed platelet suspensions were then prepared as described in section 3.2 and platelet aggregation was induced by collagen (10 μg/ml). After aggregation, platelet pellets were separated from releasates and the releasates were used for corresponding Matrigel capillary forming angiogenesis assays.

Ninety-six-well cell culture plates were coated with 50 µl of a mixture of 40 µl Matrigel (BD Biosciences, San Jose, CA, USA) diluted with 80 µl of platelet releasates from PKC inhibited platelets. Matrigel was then allowed to solidify at 37 C for 30 min. HMVECs were detached using trypsin–EDTA solution, washed and resuspended in Clonetics EBM-2 medium (Lonza Inc. Walkersville, MD, USA), and added to Matrigel-coated wells (10,000 cells per well in 100 µl media). Capillary-like structures were allowed to form for 24 hours at 37 C in a 5% CO_2 humidified atmosphere. Wells were observed and photomicrographs taken at 12 hours and 24 hours after plating using an Olympus CKX41 microscope phase-contrast microscope (Olympus America Inc., Melville, NY, USA) equipped with a digital camera. Each platelet releasate was assayed in duplicate (Jurasz et al., 2003a). Angiogenesis was quantified by measuring the area occupied by capillary-like structures using the image analysis software ImageJ (National Institutes of Health, Bethesda, MD, USA).

3.9 Immunofluorescence microscopy

Platelets aggregated with collagen that had reached an aggregation stage of 25% light transmittance in the lumi-aggregometer in presence or absence of cPKC inhibitors were fixed for 20 minutes in suspension by the addition of 1:1 vol of 8% formaldehyde. Solutions of fixed platelets in suspension were placed on polylysine-coated coverslips, which were centrifuged at 250g for 5 minutes to attach the cells to the coverslip. Platelets then were permeabilized with Tyrode's Buffer containing 0.1% Triton X-100 solution for 20 minutes. Specimens were blocked overnight in phosphate-buffered saline (PBS) with 5% BSA, then incubated with primary antibody for 2 hours, washed 3 times with PBS, and treated with appropriate secondary antibody for 2 hours, and then washed 3 times with PBS. Rabbit polyclonal antibody to PKCα IgG and rabbit polyclonal antibody to PKCβ IgG primary antibodies (Abcam, Cambridge, MA, USA) were used at 1:50 dilution and a mouse monoclonal anti-human CD62P AK-4 antibody

was used at 1:100 dilutions in PBS containing 5% BSA. Secondary antibodies DyLight 488 donkey anti-rabbit IgG and DyLight 549 goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) were used at 1:500 dilutions in PBS containing 5% BSA. Antibody controls were treated in the same fashion except for exclusion of the primary antibody. Preparations were mounted in Prolong Gold Antifade solution (Invitrogen) and analyzed at room temperature on a Leica TCS SP5 microscope equipped with a 100×/1.4 NA objective. Electronic shutters and image acquisition were under the control of Leica LAF AS software. Images were acquired by fluorescence microscopy with an image capture time of 100 to 400 ms.

3.10 Statistical analysis

For the analysis of differences between groups of data, one-way ANOVA was performed followed by Dunnett test using GraphPad Prism 5.0 (GraphPad Software). Also, paired and unpaired t tests were performed where appropriate. Data are expressed as the means ± Standard Error of the Mean (SEM). N equals the number of independent experiments. A p value of < 0.05 was considered statistically significant.

4. RESULTS

4.1 Effects of cPKC Inhibition on Platelet Aggregation

Recent evidence suggests that the conventional PKC isoforms α and β are respectively responsible for signaling α-granule release and aggregation (Konopatskaya et al., 2009; Buensuceso et al., 2005; Yoshioka et al., 2001). To investigate whether it is possibile to uncouple α-granule release from platelet aggregation via selective PKC inhibition, the effects of conventional PKC inhibitors on platelet aggregation were investigated first. Platelets were pre-incubated with the inhibitor of conventional PKC isoforms Gö 6976 (IC_{50} PKCα: 2.3 nM vs. PKCβ: 6.2 nM) at different concentrations ranging from 0 to 300 nM or DMSO as vehicle control. Platelet aggregation was then stimulated with collagen (10 µg/ml). Gö 6976 inhibited platelet aggregation in a concentration dependant manner. At the maximum concentration used of 300 nM platelet aggregation was significantly reduced to 18 ± 7% compared to control 83 ± 4% ($P < 0.05$) (Figure 4). At a lower concentration of 100 nM, platelet aggregation was significantly reduced to 55% ±10 compared to control. However at a concentration of 50 nM of Gö 6976 platelet aggregation was not significantly inhibited compared to control (77% ±8 vs. 83 ± 4%, $P > 0.05$).

Another cPKC inhibitor more selective for PKCα than PKCβ, Ro 32-0432 (IC_{50} PKCα: 9 nM vs. PKCβ: 28 nM) (0 to 10 µM), similar to Gö 6976, inhibited platelet aggregation in concentration-dependent manner (Figure 5). Significant inhibition was observed at 10 µM of Ro 32-0432 (24 ± 9% vs. control 83 ± 6%). The similar actions of these two agents are consistent with their similar structural target on the PKC enzyme. Both inhibitors target the ATP binding domain decreasing the enzyme activity.

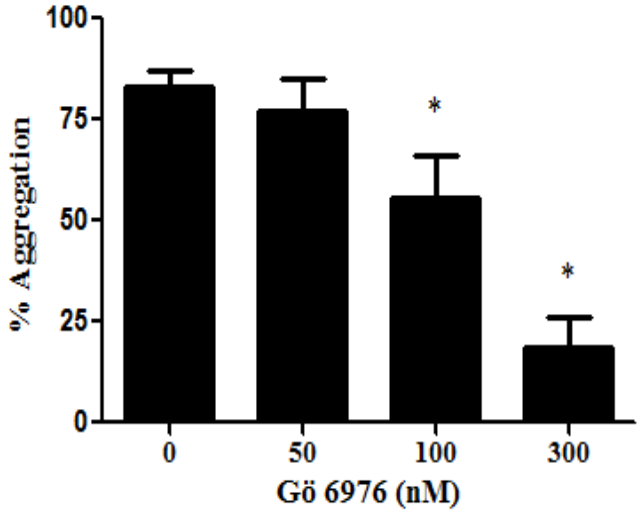
The permeable peptide cPKC inhibitor (myr-FARKGALRQ) was used at concentrations 0-1 µM but no inhibitory effect on platelet aggregation was

observed (Figure 6). Concentrations of myr-FARKGALRQ higher than 10 μM were not used in our studies because they potentiated collagen-induced aggregation. The myr-FARKGALRQ peptide targets the pseudosubstrate region of the cPKC and instead of competing for ATP binding competes with substrate binding.

Platelets were also titrated with the alleged PKCδ inhibitor Rottlerin (0-30 μM). Rottlerin inhibited platelet aggregation in a concentration-dependent manner starting at 10 μM (48.8 ± 12.3% vs. control 90.8 ± 3.7%). At a concentration of 30 μM platelet aggregation was almost completely blocked at 14.6 ± 0.9% (Figure 7A). The PKCβ inhibitor ((3-(1-(3- Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione) was also used and showed that it only inhibits aggregation at 30 μM (25.6 ± 8.8% vs. control 81.4 ± 1.5%) **(**Figure 7B).

The effect of cPKC inhibitors on platelets aggregated with the agonist thrombin (0.3 U/ml) was also measured. The inhibitor Gö 6976 (Figure 8A) and the peptide myr-FARKGALRQ (Figure 8B) did not have any effect on thrombin-induced aggregation.

A



B

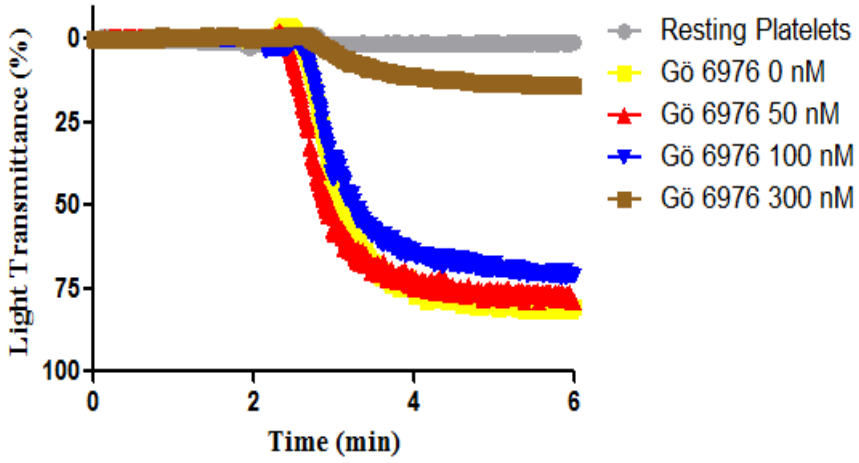


Figure 4: (A and B) Summary data and representative traces of human platelets aggregated with collagen (10 µg/ml) in the presence of Gö 6976 (0-300 nM). Gö 6976 (0-300 nM) inhibited platelet aggregation in a concentration dependant manner with maximum inhibition occurring at 300 nM (22.57 ± 9.2% aggregation).

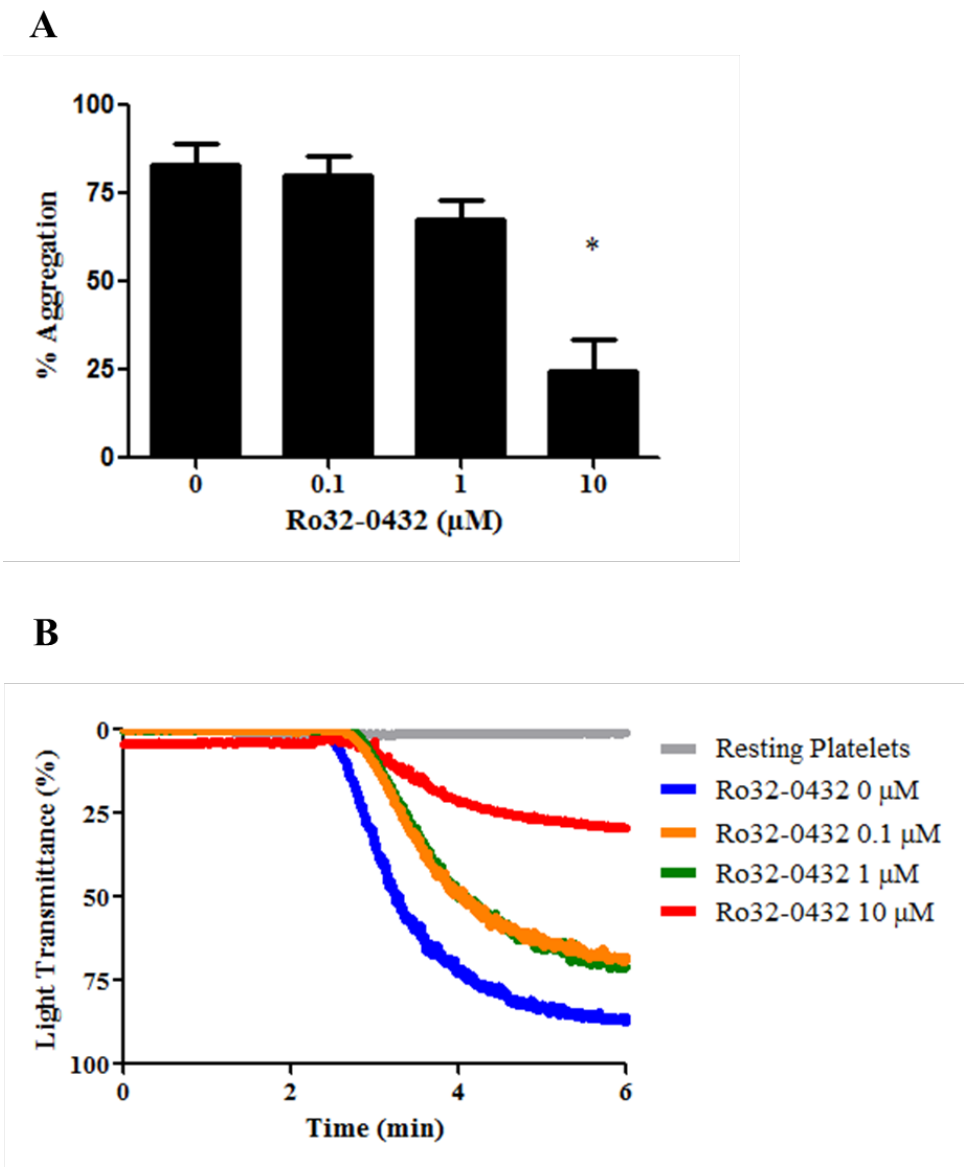


Figure 5: (A and B) Summary data and representative traces of human platelets aggregated with collagen (10 µg/ml) in the presence of Ro 32-0432 (0-10 µM). Ro32-0432 inhibited platelet aggregation in a concentration dependent manner N=5 *, $P < 0.05$

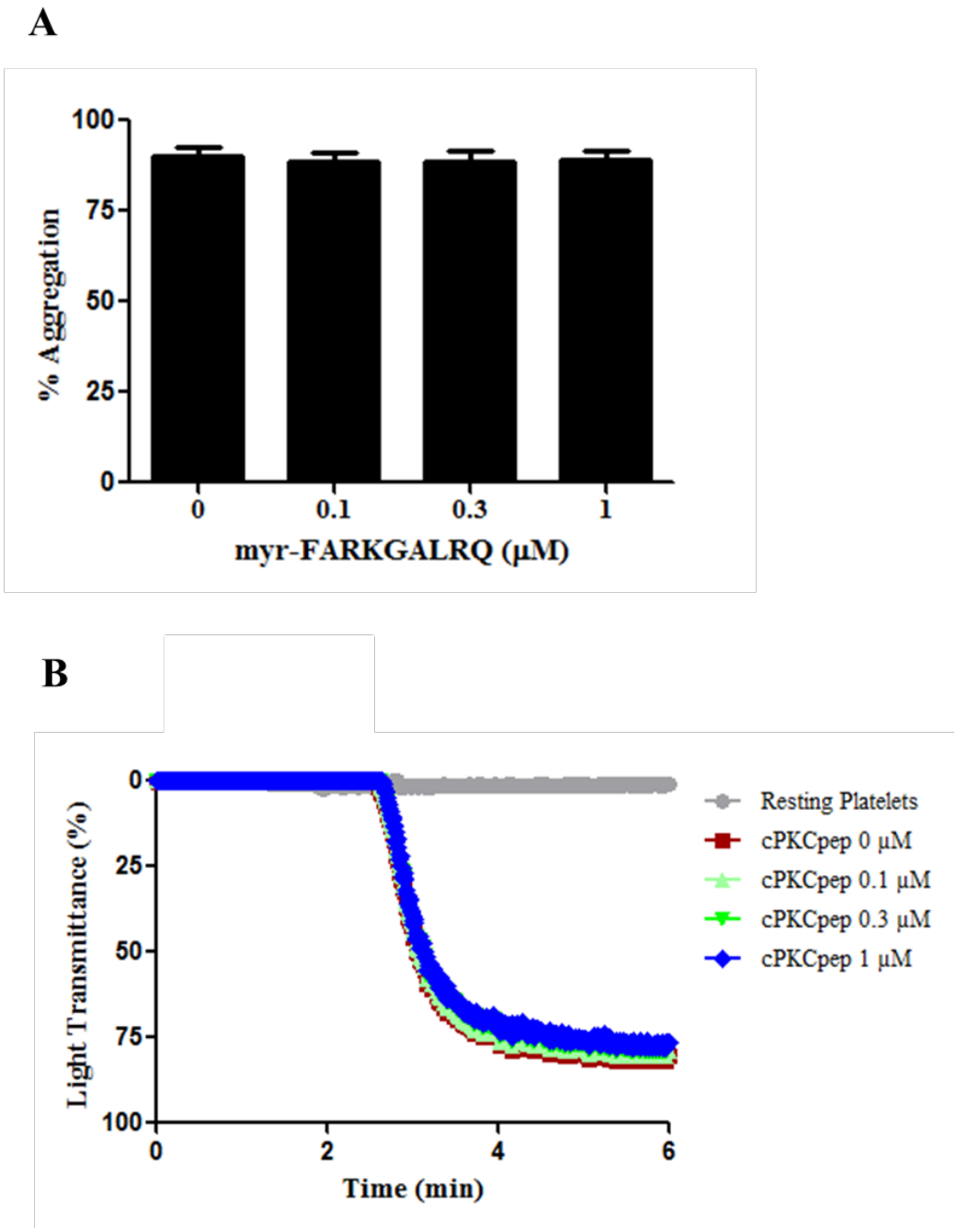


Figure 6: (A and B) Platelets aggregated with collagen in the presence of peptide cPKC inhibitory peptide (myr-FARKGALRQ) (0-1 µM). The peptide does not inhibit platelet aggregation. N= 9. *, $P < 0.05$.

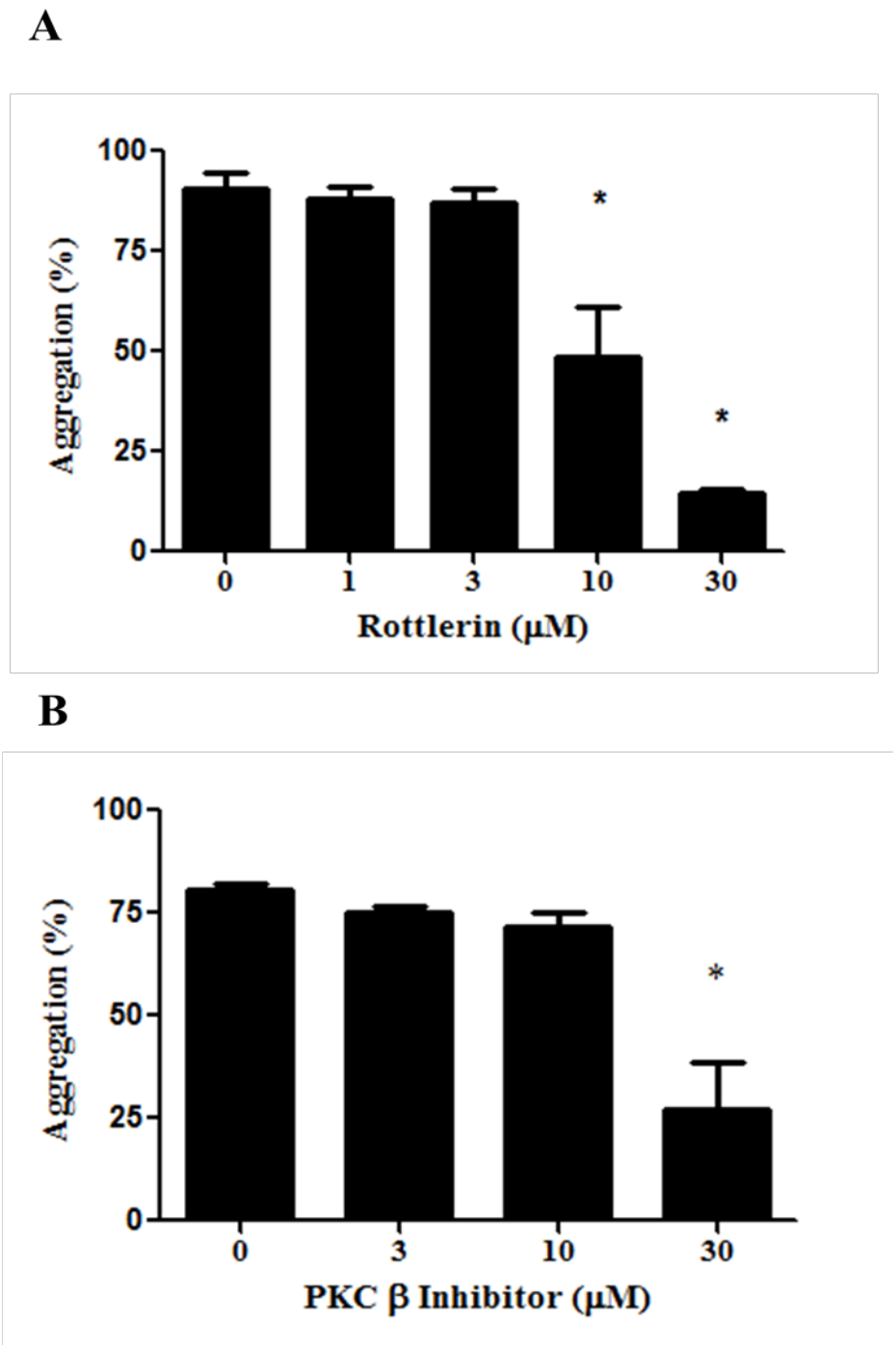


Figure 7: Summary data of human platelets aggregated with collagen (10 µg/ml) in the presence of **(A)** Rottlerin (0-30 µM) and **(B)** PKCβ Inhibitor (0-30 µM). Rottlerin inhibited platelet aggregation in a concentration dependent manner with maximum inhibition occurring at 30 µM (14.7 ± 1.2% aggregation). PKCβ Inhibitor inhibited platelet aggregation at 30 µM. N=5 *, $P < 0.05$.

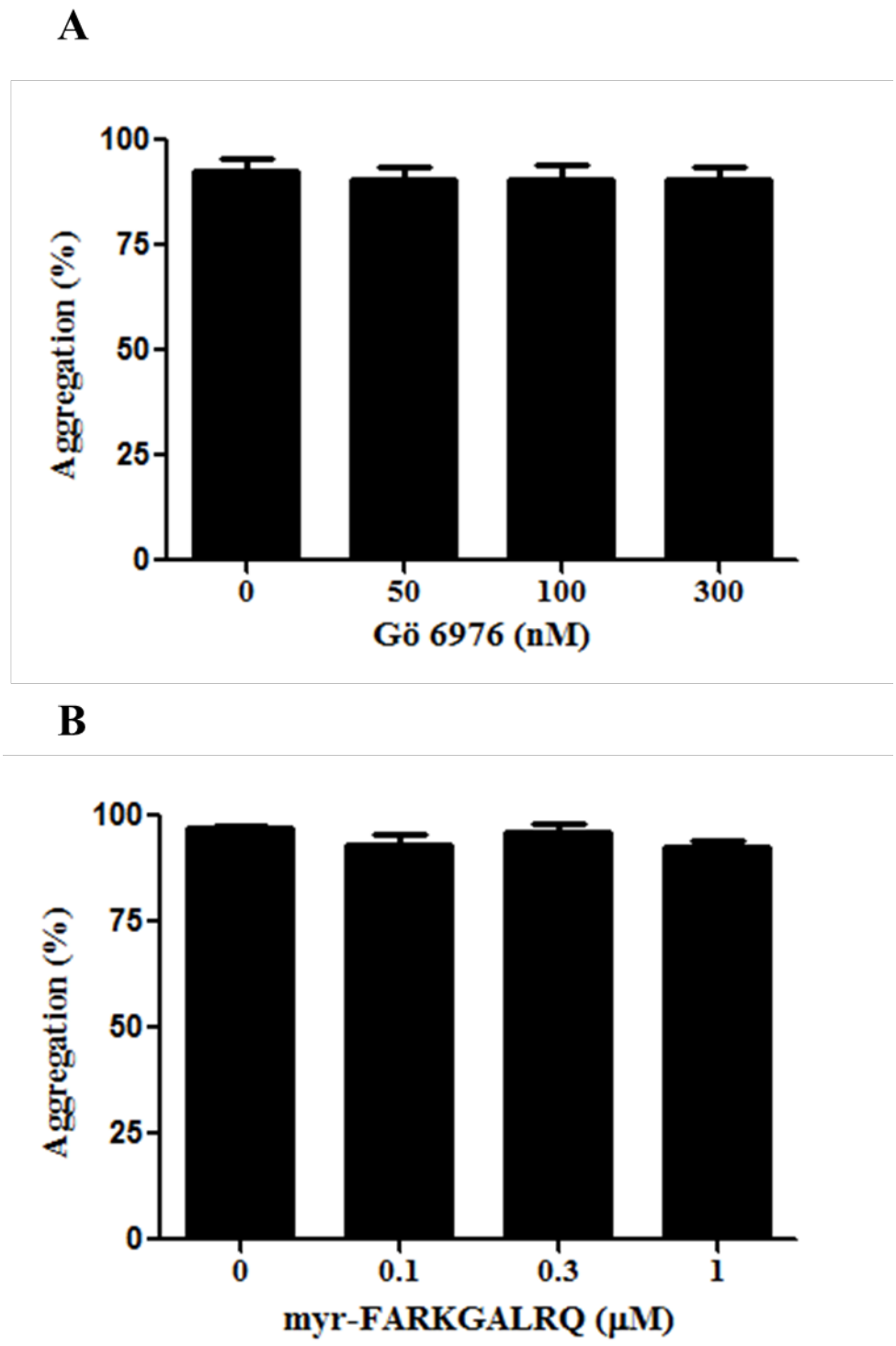


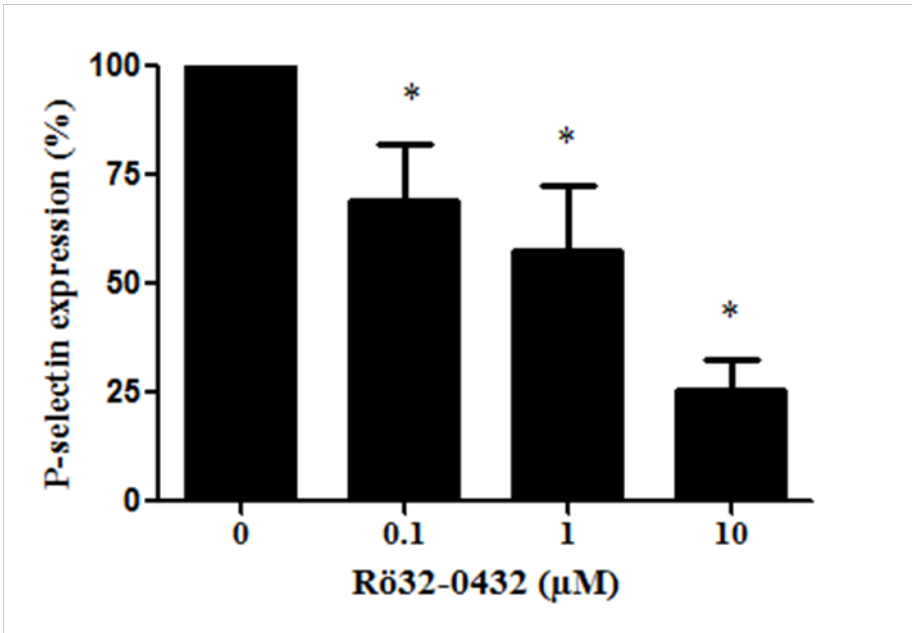
Figure 8: Summary data of human platelets aggregated with thrombin (0.3 U/ml) in the presence of **(A)** Gö 6976 (0-300 nM) and **(B)** myr-FARKGALRQ) (0-1 µM). No effect was observed by both cPKC inhibitors on platelet aggregation. N=5 *, $P < 0.05$

4.2 Effect of cPKC on PKC inhibition on α-granule secretion

To investigate the effects of cPKC inhibitors on α-granule release, the surface exposure of P-selectin, a receptor present in within α-granules, was measured as a marker of α-granule release. P-selectin is exposed on to the outer platelet membrane upon α-granule release and aids in cell-cell interaction between platelets, leukocytes and endothelial cells. P-selectin exposure was measured by flow cytometry as quick screen of α-granule release. In the presence of Ro 32-0432, P-selectin exposure was reduced significantly at 10 μM (29.3 ± 8% vs. control 100%, $P < 0.05$). At submaximal concentration of Ro 32-0432 (1 μM) the expression of P-selectin was reduce to 57.8 ± 14.5% vs. control 100% ($P < 0.05$) (Figure 9). The results with Ro 32-0432 are comparable to results previously obtained from our lab with Gö 6976. At 300 nM of Gö 6976, P-selectin surface exposure was almost completely abolished (17 ± 2.5% vs. control 100%, $P < 0.05$). Interestingly at sub-maximal concentrations 30-200nM of Gö 6976 also significantly reduced P-selectin surface exposure (53.4 ± 5.7% vs. control 100%, $P < 0.05$). Experiments by members of our lab using the peptide inhibitor myr-FARKGALRQ had no effect on platelet aggregation but reduced P-selectin expression at 1 μM (32.2 ± 22.8 % vs. control 100%, $P < 0.05$), thus uncoupling α-granule release from aggregation.

The PKCβ inhibitor (Figure 10) inhibited P-selectin surface exposure in a concentration-dependent manner with significant inhibition occurring at 30 μM (41.9 ± 9.3% vs. control 100%, $P < 0.05$).

A



B

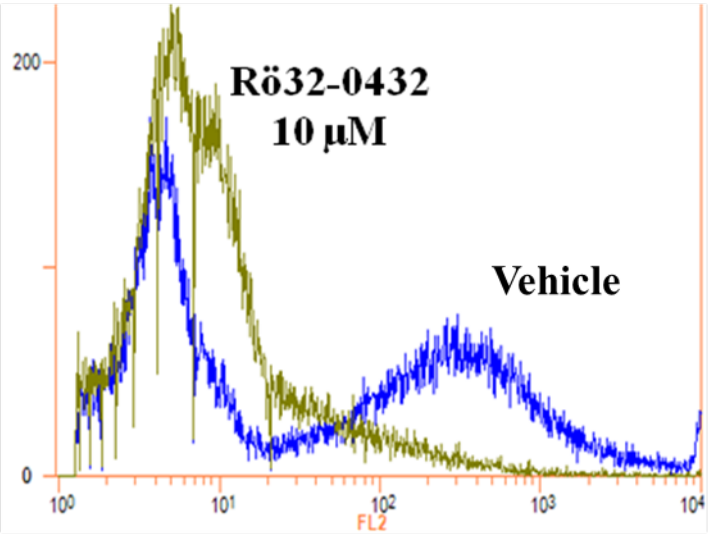


Figure 9 (A and B): Bar Graphs and Histograms of platelet P-selectin surface exposure as measured by flow cytometry. Rö32-0432 at 10 µM inhibits P-selectin expression. N= 4. *, P < 0.05 vs vehicle.

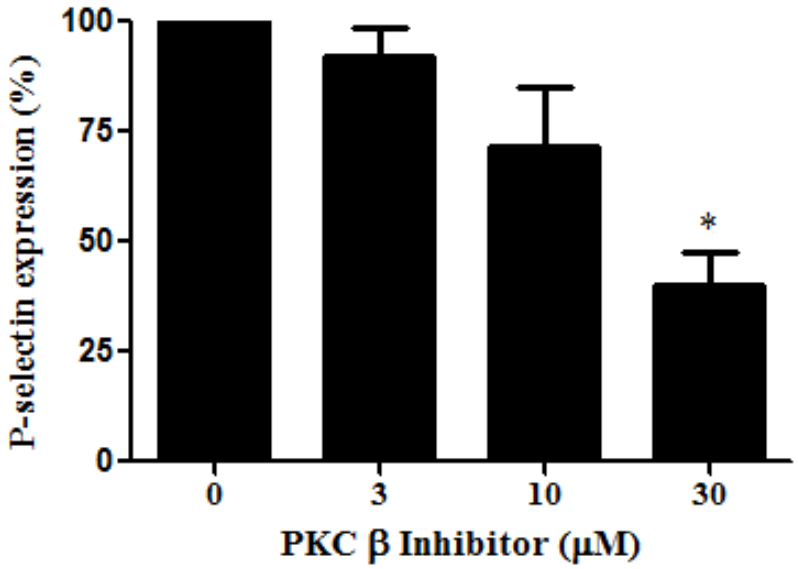


Figure 10: Bar graph of platelet P-selectin surface exposure as measured by flow cytometry. PKCβ Inhibitor at 30 μM inhibits P-selectin expression. N=3. *, $P < 0.05$ vs vehicle.

4.3 Effect of Inhibitors on cPKC Localization

At a resting state platelets have their granules scattered throughout the platelet, while upon aggregation, platelets concentrate their granules at their centers' for release (Flaumenhaft, 2003). To determine if conventional PKC inhibitors at concentrations which uncouple platelet aggregation from α-granule release interfere with PKCα or β signaling, we investigated the effects of 1 μM Ro 32-0432 on PKCα and β colocalization to these centralized granules following activation by collagen.

In resting platelets the PKCα was primarily found to localize to the plasma membrane (Figure 11A), while the PKCβ throughout the platelet cytosol (Figure 11D). P-selectin immunofluorescence presented a punctuated pattern consistent with its localization within α-granules. Upon collagen activation PKCα translocated to the platelet center and colocalized with P-selectin in individual activated platelets and small aggregates composed of 2-3 platelets, indicating PKCα targeting to the α-granule (Figure 11B inset). PKCβ did not translocate to the centers of platelets upon activation by collagen (Figure 11E). Ro 32-0432 (1 μM) inhibited collagen-induced PKCα translocation to platelet centers and its association with α-granules, which resulted in P-selectin remaining un-released from the central granular cores of many platelets (Figure 11C). In contrast Ro 32-0432 did not have any apparent effect on PKCβ localization (Figure 11E). Even in very large aggregates that grew to include hundreds of platelets, Ro 32-0432 (1 μM) prevented PKCα targeting to and P-selectin release from α-granules (Figure 12).

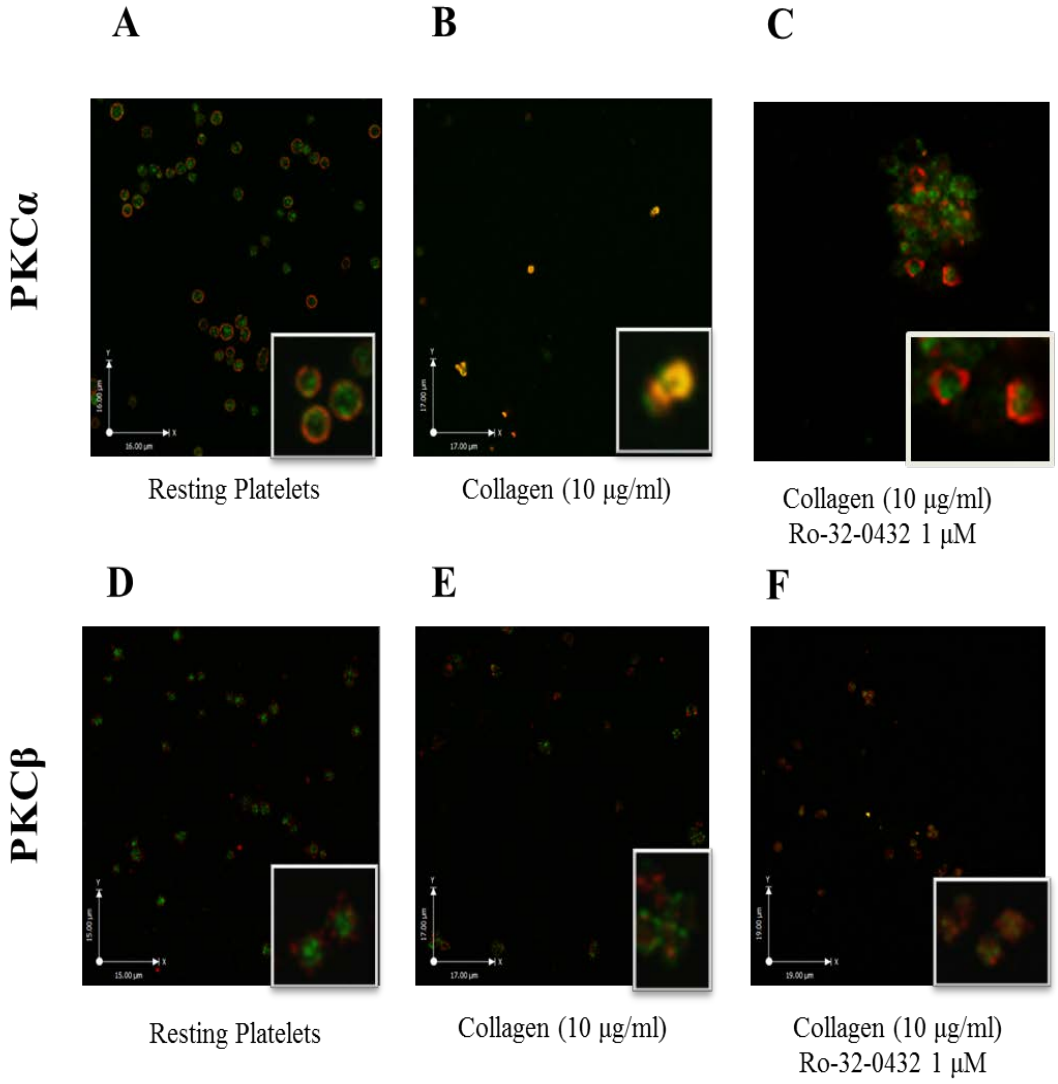
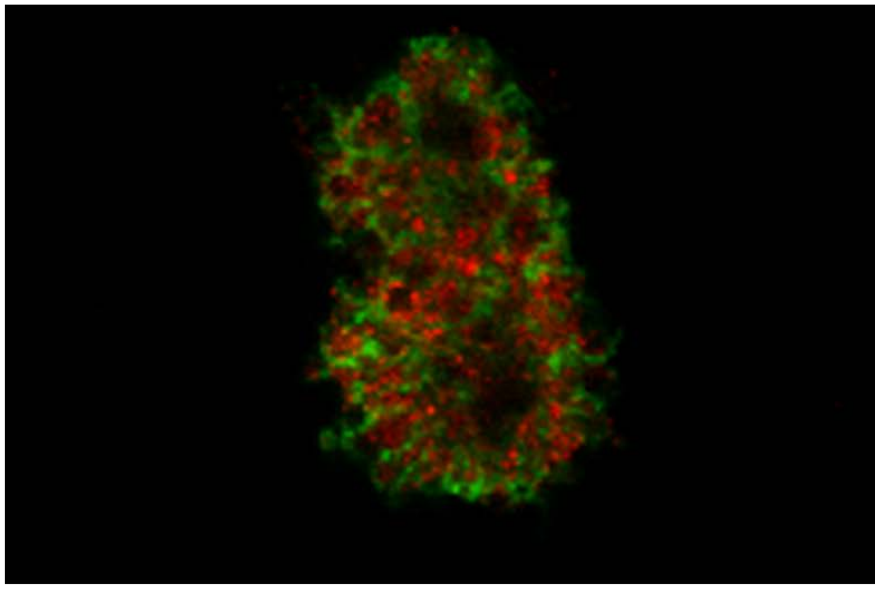


Figure 11: Immunofluorescence confocal microscopy of resting platelets **(A and D),** collagen-aggregated control **(B and E)** and Ro 32-0432 1 µM inhibited platelets of **(C and F)** demonstrating the localization of PKCα (A, B and C), PKCβ (D, E and F) and P-selectin (A-F). PKCα and PKCβ both are represented by red fluorescence and P-selectin presents green fluorescence. Representative images are from three independent experiments using a Leica TCS SP5 microscope equipped with a 100×/1.4 NA objective. Scale bars represent 16 µm. The inset shows a zoom-in on single platelets.

A



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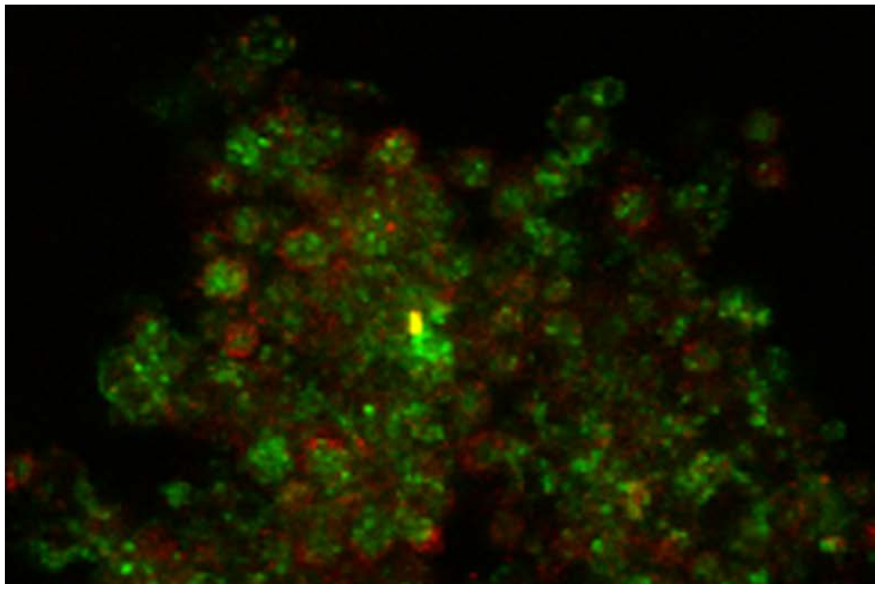


Figure 12: Immunofluorescence microscopy demonstrating the PKCα translocation inhibitory effects of Ro 32-0432 (1 μM) in large aggregates of collagen-stimulated platelets (B) compared to control (A). PKCα is represented by red fluorescence and P-selectin by green fluorescence. Representative images are from three independent experiments using a Leica TCS SP5 microscope equipped with a 100×/1.4 NA objective.

4.4 Effect of cPKC Inhibition on the Release of Angiogenic Regulators

To examine the effects of conventional PKC inhibition on the release of the angiogenic regulators from α-granules, concentrations of VEGF and TSP-1 were determined in the releasates of collagen-aggregated platelets incubated with different cPKC inhibitors or vehicle. The pro-angiogenic molecule vascular endothelial growth factor (VEGF) and the anti-angiogenic molecule thrombospondin-1 (TSP-1) were measured using commercially available ELISA kits.

Platelet inhibition by Gö 6976 decreased the levels of VEGF released from platelets in a concentration-dependent manner (Figure 13A). Gö 6976 at concentrations of 100 nM and 300 nM exerted significant inhibitory effects on VEGF (100.0 ± 0.0% control vs. 61.9 ± 12.8% vs. 28.5 ± 9.7%, $P < 0.05$) released from aggregating platelets. TSP-1 release in response to collagen was also significantly inhibited at concentrations of 100 nM and 300 nM (100.0 ±0.0% control vs. 61.7 ± 9.9% vs. 17.2 ± 6.4%, $P < 0.05$). The levels of the pro-angiogenic regulator bFGF in collagen-aggregated releasates were also measured; however the levels in platelet releasates are difficult to measure using commercial ELISA kits since bFGF is present in low amounts in platelets and the bFGF found in platelet releasates is at the lower limit of detection by ELISA. For example values of bFGF released by platelets stimulated by collagen (10 μg/ml) were 1.29 ± 0.8 pg/ml while according to the kit manual the minimum detectable dose of FGF basic is typically less than 3 pg/mL. Despite this problem bFGF release is likely impaired by Gö 6976. At 300 nM in particular bFGF levels were reduced to (100.0 ±0.0% control vs. 23.7 ± 23.6%) (Figure 13C).

The small molecule inhibitor Ro 32-0432 inhibited platelet VEGF release starting at a concentration of 1 μM (100.0 ± 0.0% control vs. 47.3 ± 23.6%, $P < 0.05$), a concentration which did not significantly inhibit aggregation. In contrast, at the same concentration (1 μM), Ro 32-0432 failed to inhibit the release of TSP-1 from collagen-aggregated platelets but did so at 10 μM (Figure 14).

The peptide inhibitor myr-FARKGALRQ (1 μM) also significantly reduced VEGF release from collagen aggregated platelets (100.0 ± 0.0% control vs. 55.2 ± 7.6%, $P < 0.05$); however, the release of TSP-1 was not affected by the peptide inhibitor (100.0 ± 0.0% vs. 90.3 ± 8.4%, $P < 0.05$) (Figure 15). Both, the peptide inhibitor myr-FARKGALRQ (1 μM) and Ro 32-0432 (1 μM) both reduced the release of the pro-angiogenic molecule VEGF from platelets without affecting the release of the anti-angiogenic molecule TSP-1; suggestive of a down-regulation of the overall pro-angiogenic capabilities of platelet releasates.

The putative PKCδ inhibitor Rottlerin also preferentially inhibited VEGF over TSP-1 release as some of the conventional PKC inhibitors. However, unlike Ro 32-0432, Rotterlin, at a high concentration (30 μM) which almost completely blocked platelet aggregation (14.6 ± 0.9% vs. 90.8 ± 3.7% control, $P < 0.05$) had a inhibitory effect on VEGF (100.0 ± 0.0% vs. 44.6 ± 7.7%, $P < 0.05$) but not TSP-1(100.0 ± 0.0% vs. 97.8 ± 2.1%, $P > 0.05$) release (Figure 16). The results obtained with Rottlerin are comparable to those obtained with myr-FARKGALRQ

Similar to the effects on thrombin-induced aggregation conventional PKC inhibitors Gö 6976 and myr-FARKGALRQ failed to inhibit VEGF or TSP-1 release from thrombin-aggregated platelets (Figure 17 and Figure 18).

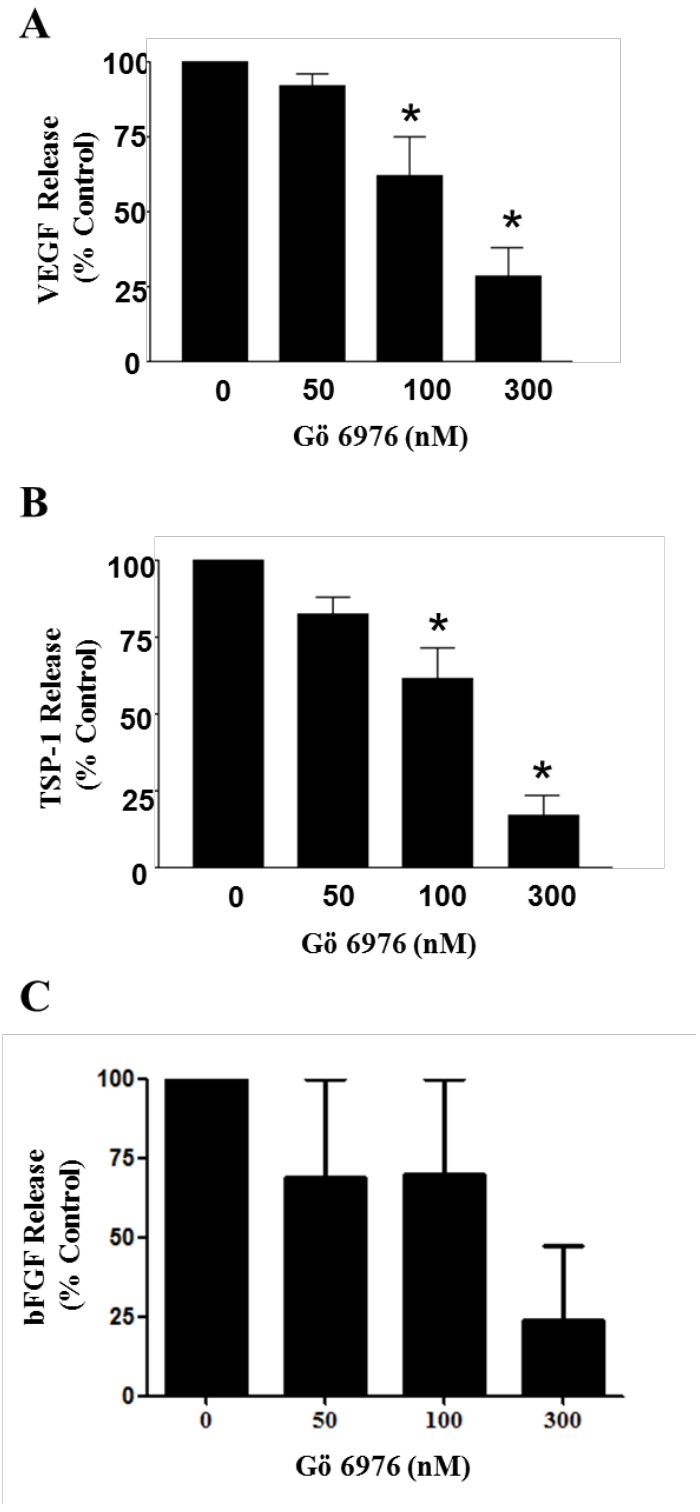


Figure 13: Experiments demonstrating the inhibition in (A) $VEGF_{165}$, (B) TSP-1 and (C) bFGF release from platelets by increasing concentrations of Gö 6976. Aggregation was induced by collagen (10 μg/ml) N= 9, *, $P < 0.05$ vs. collagen-aggregated platelets in the absence of Gö 6976

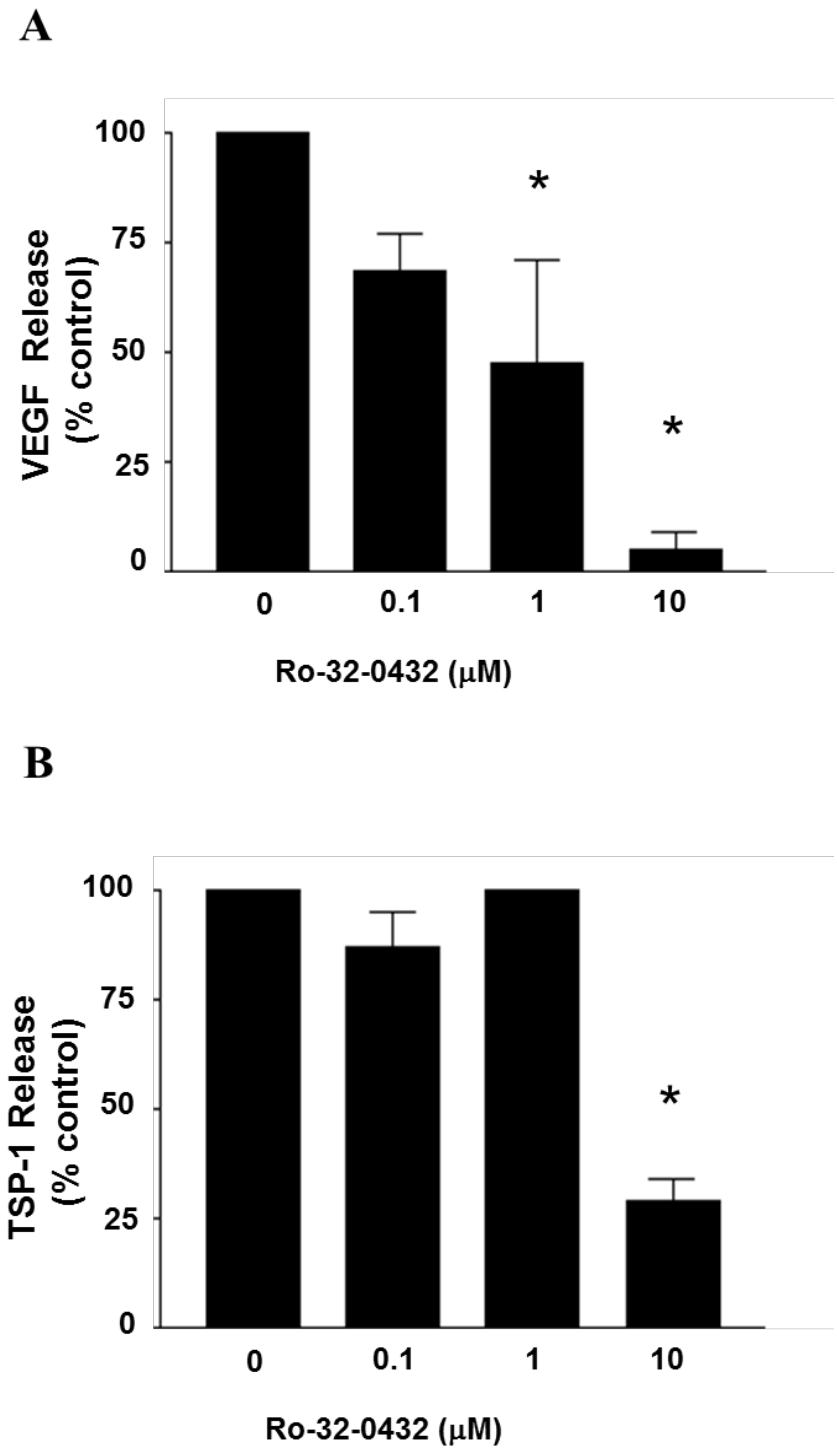


Figure 14: Experiments demonstrating the inhibition in (A) $VEGF_{165}$ and (B) TSP-1 release from platelets at increasing concentrations of Ro 32-0432. Release of $VEGF_{165}$ was inhibited at 1 and 10 μM, while TSP-1 release was inhibited at 10 μM. Aggregation was induced by collagen (10 μg/ml) N= 5, *, $P < 0.05$ vs. collagen-aggregated platelets in the absence of Ro 32-0432.

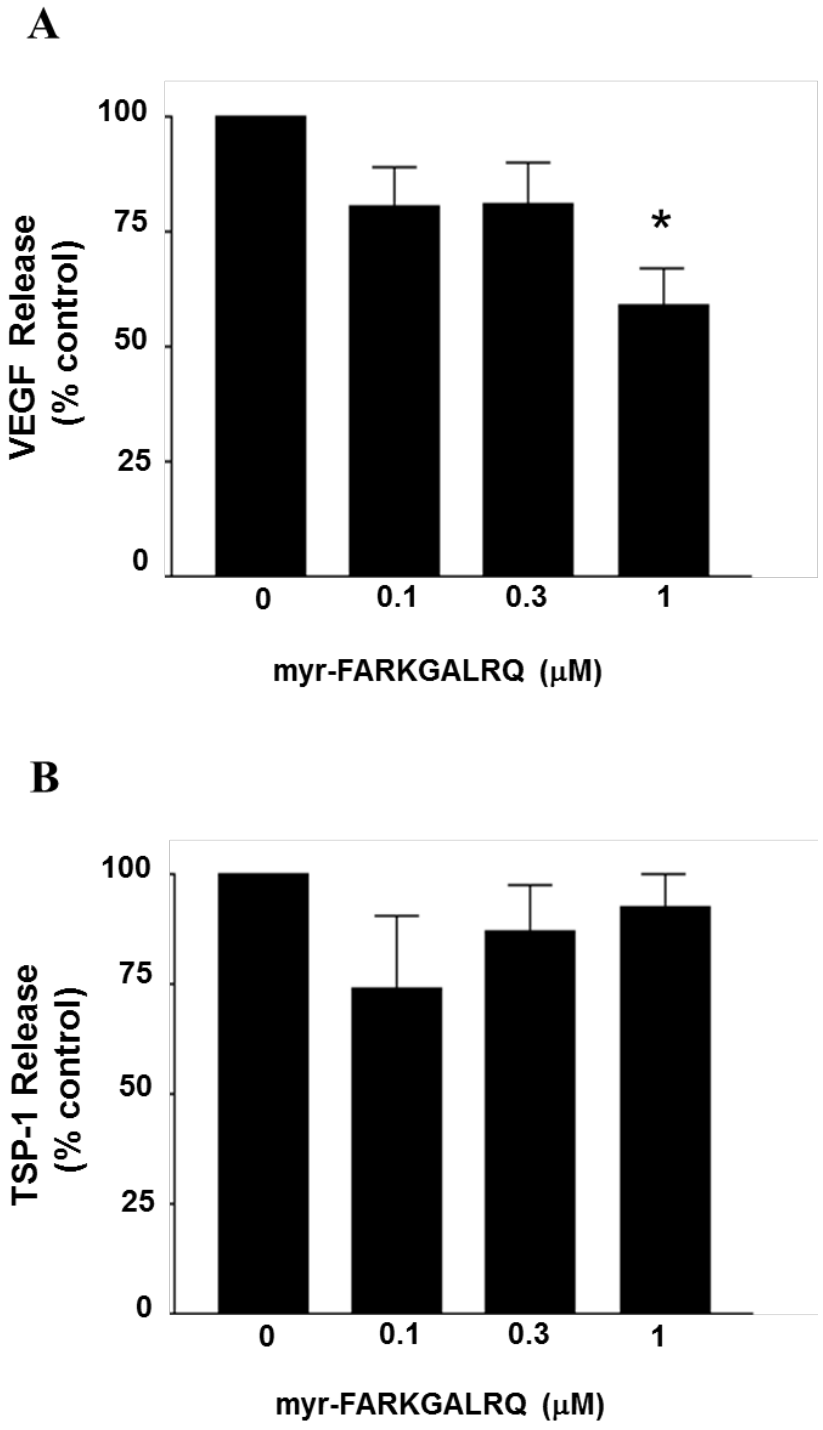


Figure 15: Experiments demonstrating the effects of increasing concentrations of myr-FARKGALRQ on (A) $VEGF_{165}$ and (B) TSP-1 release from platelets. . Release of $VEGF_{165}$, but not TSP-1, from α-granules was inhibited by myr-FARKGALRQ (1 μM). Aggregation was induced by collagen (10 μg/ml). N= 9, *, $P < 0.05$ vs. collagen-aggregated platelets in the absence of myr-FARKGALRQ.

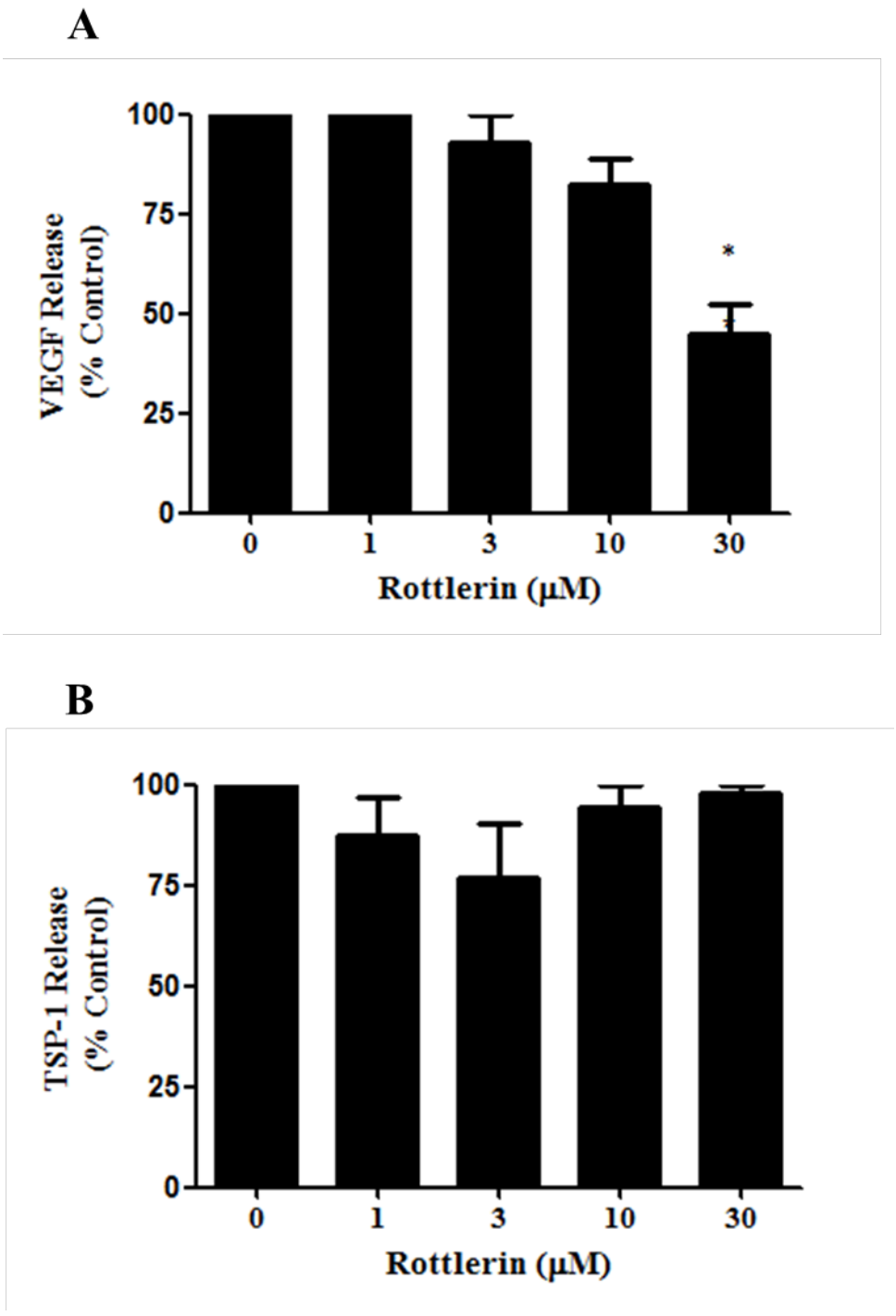


Figure 16: Experiments demonstrating the inhibition in (A) $VEGF_{165}$ and (B) TSP-1 release from platelet with increasing concentrations of Rottlerin. Release of $VEGF_{165}$ was inhibited at 10 and 30 µM. N= 5, *, $P < 0.05$ vs. control collagen aggregated platelets in absence of Rottlerin.

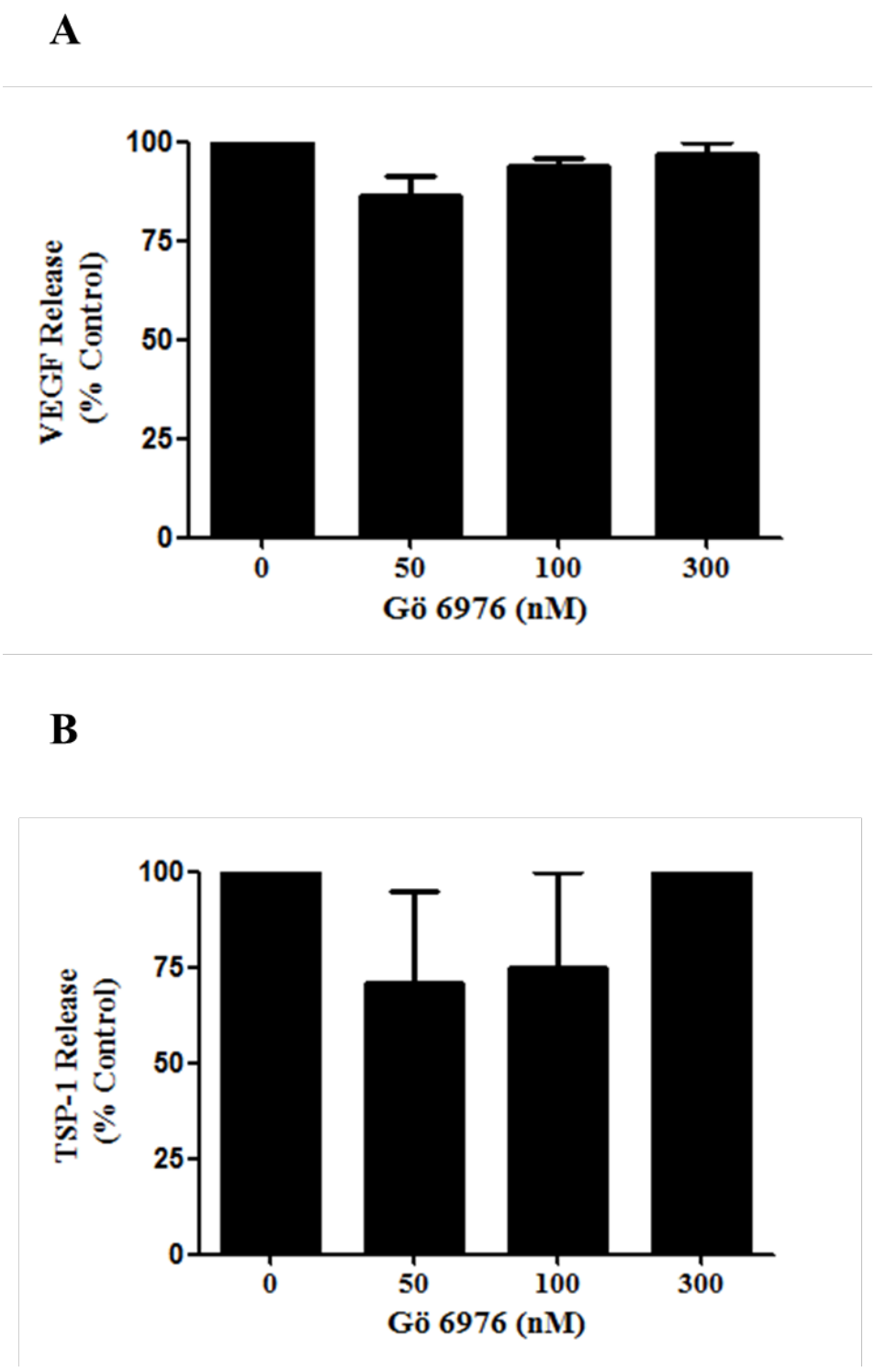


Figure 17: (A) $VEGF_{165}$ and (B) TSP-1 release from thrombin (0.3 U/ml) aggregated platelets at increasing concentrations of Gö 6976. No significant inhibition was observed. N= 5.

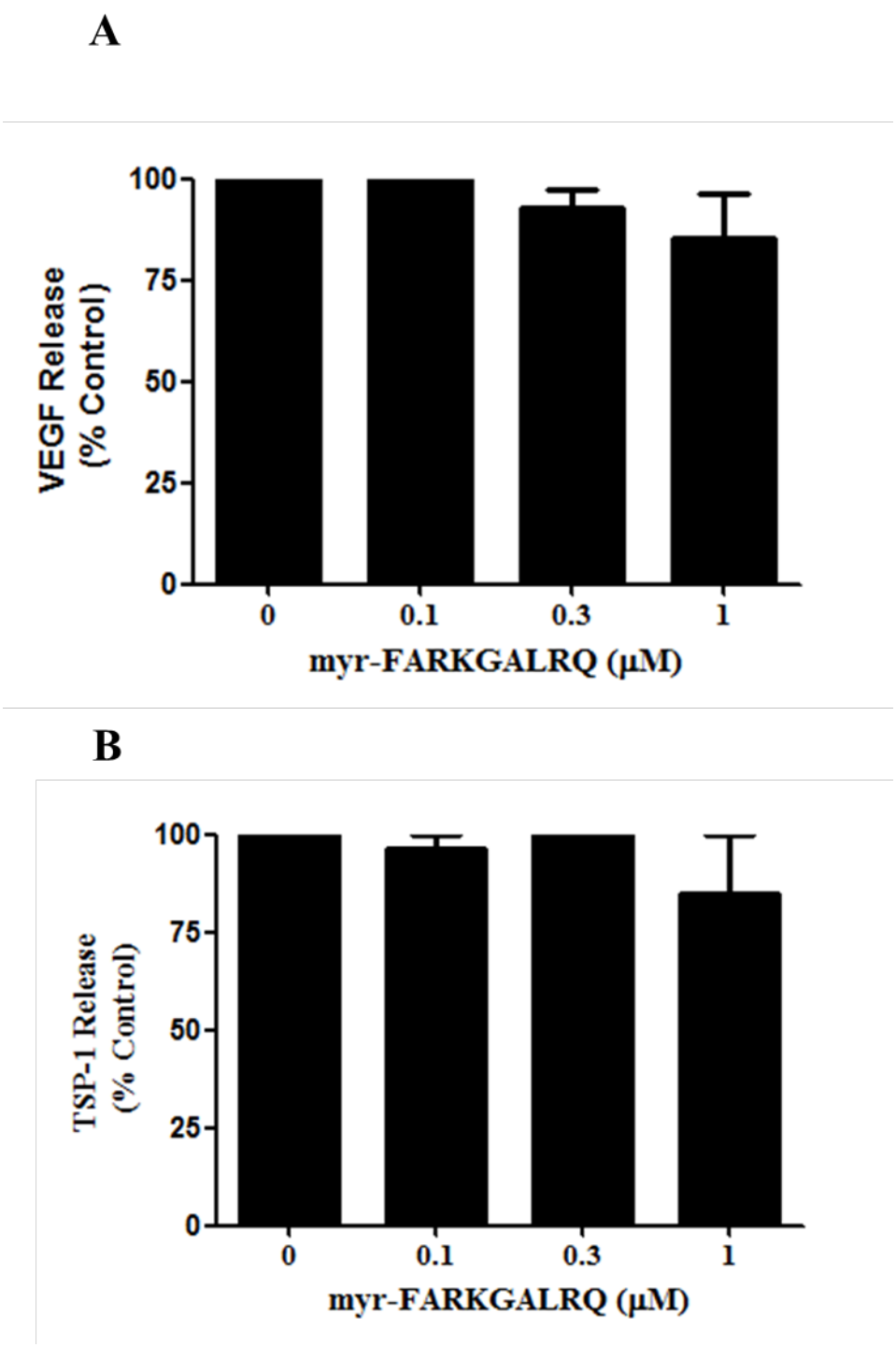


Figure 18: (A) $VEGF_{165}$ and **(B)** TSP-1 release from thrombin (0.3 U/ml) aggregated platelets at increasing concentrations of myr-FARKGALRQ. No significant inhibition was observed. N= 5.

4.5 Effect cPKC inhibition on Dense Granule Release

PKCα has been also reported to be involved in the regulation of dense granule release (Yoshioka et al., 2001; Konopatskaya and Poole, 2010b), so the effect of cPKC inhibitors on dense granule release was also investigated. The ATP released from dense granules was measured as a marker of dense granule release and was quantified using Lumi-aggregometry. The release in the presence of cPKC inhibitors was expressed as percentage of control collagen aggregated platelets. Both Gö 6976 and Ro 32-0432 inhibited ATP release in a concentration response manner (Figure 19 A and B). Concentrations of Gö 6976 (100 nM) and Ro 32-0432 (1 μM), which inhibited angiogenesis regulator release from α-granules, also inhibited ATP release (72.1 ± 10.2% vs. control 100% and 57.1 ± 11.7% vs. control 100%, respectively; $P < 0.05$).

To explain why conventional PKC inhibitors may have more profound effects on α- than δ-granule release, we studied their release in response to the PKC activator phorbol myristate acetate (PMA). Platelet P-selectin expression and ATP release in response to increasing concentrations of PKC activator Phorbol myristate acetate (PMA) (0-10 μM). PMA induced maximal P-selectin expression (α-granule release) at lower PMA concentrations (EC_{50} 1.03 nM) than maximal ATP secretion (δ-granule release) (EC_{50} 125.5 nM) (Figure 20). This data offers a possible explanation for the observed differences in the effect that cPKC inhibition has on α and dense granule release

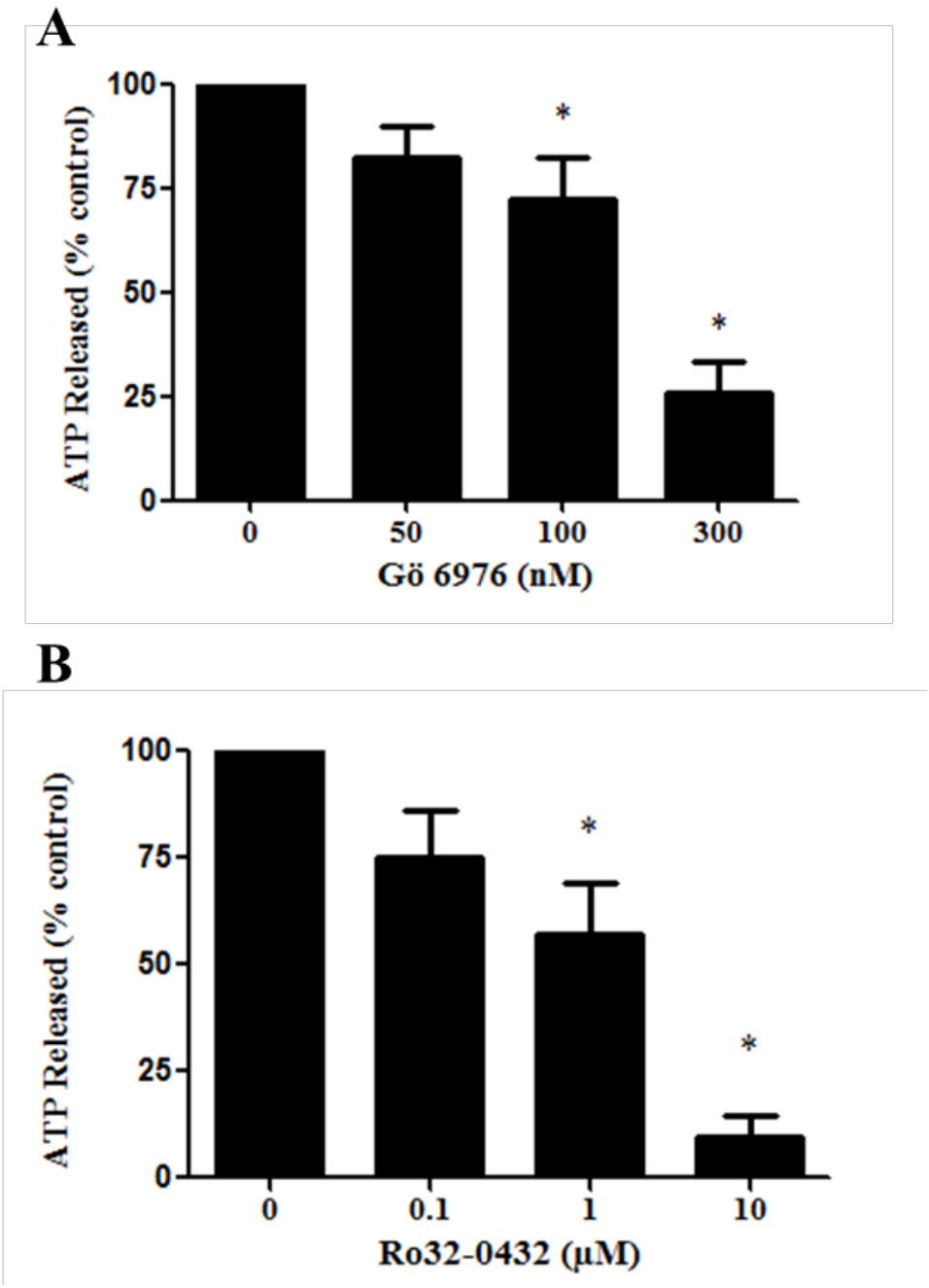


Figure 19: Platelets aggregated with collagen (10 µg/ml) in the presence of **(A)** Gö 6976 (0-300 nM) and **(B)** Ro 32-0432(0-10 µM). Dense granule secretion was measured by monitoring ATP secretion using lumi-aggregometry. N= 5, *, $P < 0.05$ vs. control collagen-aggregated platelets.

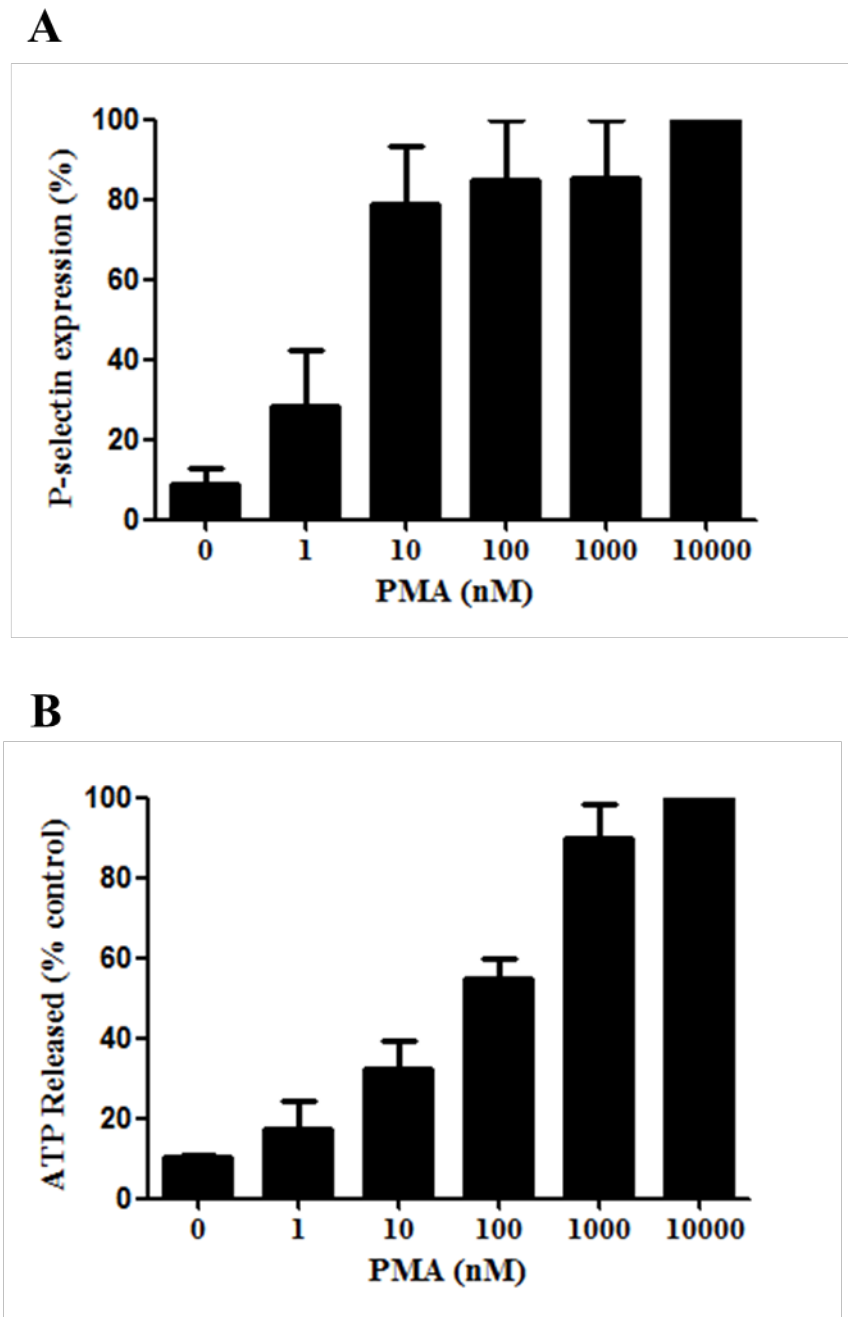


Figure 20: Platelets aggregated with increasing concentrations of PMA (0-10000 nM). **(A)** α-granule release was measured by P-selectin expression using flow cytometry. **(B)** ATP secretion was measured as a marker of dense granule release. N= 5.

4.6 Effects of PKC-Inhibited Platelet Releasates on Matrigel Capillary Tube Formation

To investigate if inhibition of angiogenesis regulator release from α-granules using cPKC inhibitors had any functional effects on platelet-stimulated angiogenesis, Matrigel® capillary tube formation assays were performed. Matrigel was diluted by releasates from collagen-stimulated platelets pretreated with Gö 6976 (100 nM), Ro 32-0432 (1μM), or vehicle prior to aggregation. HMVEC were subsequently plated on the Matrigel and formed capillary-like structures within 12 to 24 hours. Releasates from Gö 6976 (100 nM) inhibited platelets had no significant effect on platelet-stimulated angiogenesis as measured by total surface area covered by capillary tubes after 24 hours (0.738 ± 0.115 mm^2 vs. 0.726 ± 0.093 mm^2, $P > 0.05$) (Figure 21). However, compared to controls, releasates from Ro 32-0432 (1 μM) inhibited platelets (Figure 22) significantly reduced the total surface area covered by capillary tubes after 24 hours (0.777 ± 0.161 mm^2 vs. 0.490 ± 0.060 mm^2, $P > 0.05$). The decrease in capillary tube formation caused by Ro 32-0432 (1 μM) pretreated platelet releasates is consistent with results previously obtained in our laboratory with releasates from myr-FARKGALRQ (1 μM) pretreated platelets. Previously, compared to controls, releasates from myr-FARKGALRQ (1 μM) pretreated platelets also inhibited capillary tube formation on Matrigel (1.563 ± 0.022 vs. 1.397 ± 0.061, $P < 0.05$) *(unpublished data).* Interestingly, both Ro 32-0432 and myr-FARKGALRQ at 1 μM inhibit the release of the pro-angiogenesis regulator VEGF, but not the anti-angiogenesis regulator TSP-1, from platelet α-granules.

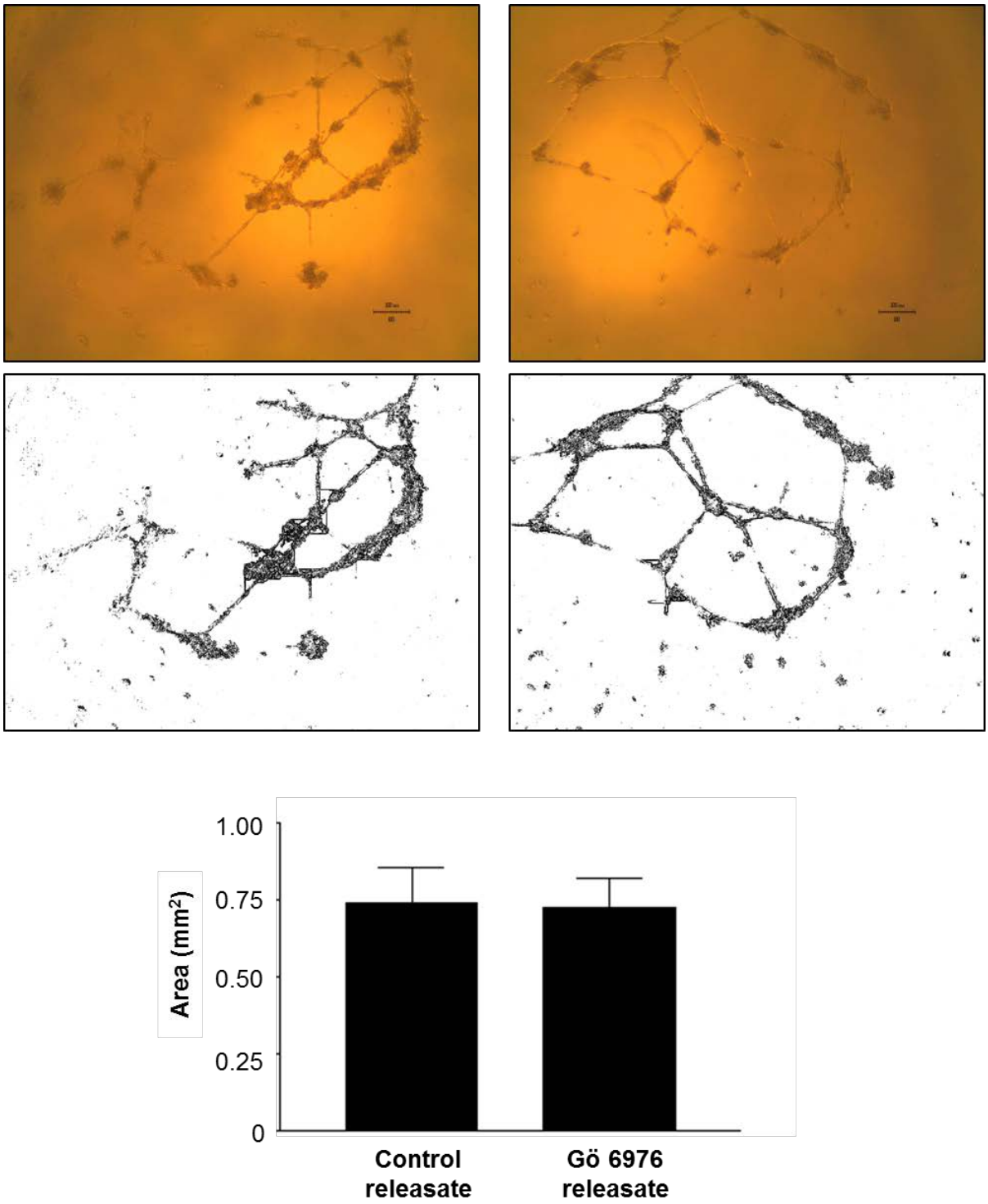
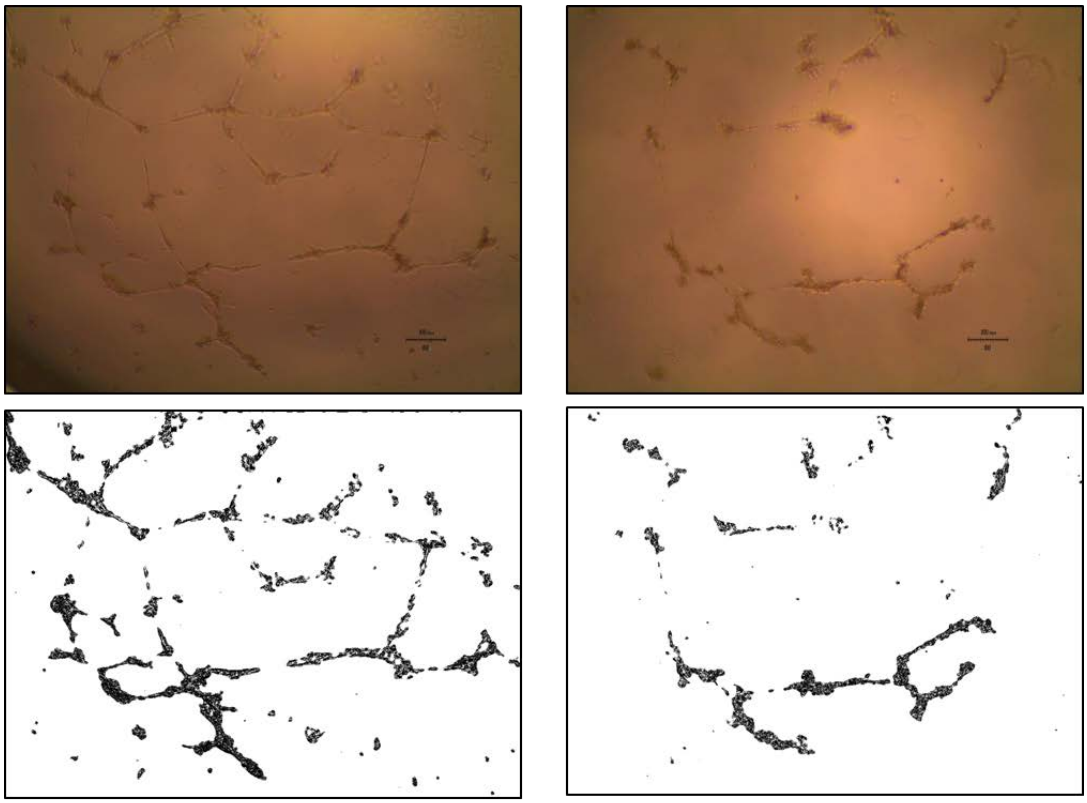


Figure 21: Photomicrograph (upper panels) and contour traces (lower panels) of HMVEC forming capillary-like tubes on Matrigel in response to releasates from control and Gö6976 (100 nM)-inhibited platelets. Platelets were aggregated by collagen (10 μg/ml). Bars represent 100 μm. Surface area was measured using ImageJ.



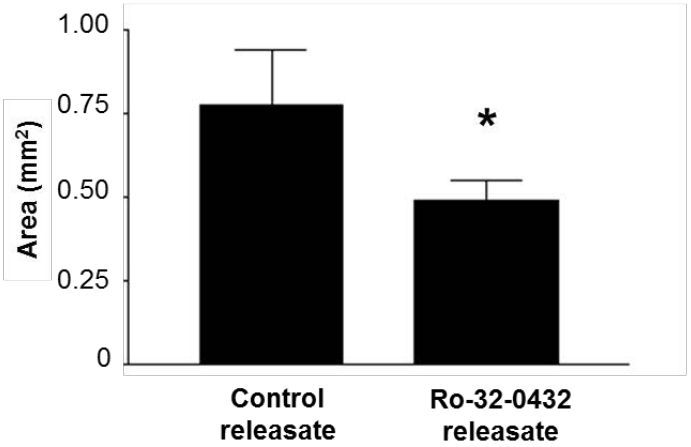


Figure 22: Photomicrograph (upper panels) and contour traces (lower panels) of HMVEC forming capillary-like tubes on a Matrigel in response to releasates from control and Ro 32-0432 (1 μM)-inhibited platelets. Platelets were aggregated by collagen 10 μg/ml. Releasates from platelets treated with Ro 32-0432 (1 μM) showed a weaker ability to promote formation of capillary-like tubes by HMVEC on matrigel compared to control. Bars represent 100 μm. Surface area was measured using ImageJ.

4.7 Effect of cPKC inhibition on Tumor Cell Induced Platelet Aggregation (TCIPA) and Angiogenesis Regulator Release

Tumor survival and metastasis are in part dependent on the interaction of tumor cells and platelets. For example platelets of tumor-bearing mice promote angiogenesis to a larger degree than platelets from mice with no tumors (Pietramaggiori et al., 2008). These observations of increased angiogenesis promotion by platelets in the presence of cancer are similar to experiments using cancer cell lines such as HT-1080, A549, and MCF-7. The different cell lines stimulate the release of angiogenesis regulators such as MMP-2, VEGF, and angiostatin from platelets, and this is correlated positively with the ability tumor cells have to induce platelet aggregation (Jurasz et al., 2001a, 2003a; Battinelli et al., 2011). Prostacyclin a potent inhibitor of platelet aggregation has been found to inhibit Tumor Cell Induced Platelet Aggregation (TCIPA) (Jurasz et al., 2001b, 2004) in contrast with the antiplatelet agent aspirin which exerted no significant effect on TCIPA (Jurasz et al., 2001a; Medina et al., 2006).

To study whether TCIPA induced by A549 lung carcinoma cells could be repressed by inhibitors of conventional PKC washed platelets were pre-incubated for 2 min with Gö 6976 (100 nM), Ro 32-0432 (1μM) and myr-FARKGALRQ (1 μM) before the addition of cancer cells (5×10^4). Conventional PKC inhibition did not have any effect whatsoever on platelet aggregation induced by A549 cells (Control 87.4 ± 7.1%; Gö 6976 88.2 ± 6.2%; Ro 32-0432 92.2 ± 4.9%; and myr-FARKGALRQ 91.4 ± 5.6%) (Figure 23A). VEGF and TSP-1 release was also measured during A549-induced TCIPA. The release of VEGF (Figure 23B) was not reduced significantly by Gö 6976 (93.6 ± 2.1%). However VEGF was reduced significantly by Ro 32-0432 to 68.3 ± 5.2% vs. control 100% ($P < 0.05$) and by myr-FARKGALRQ to 74.2 ± 12.2% vs. control 100% ($P < 0.05$). TSP-1 release (Figure 23C) was not reduced by small molecule cPKC inhibitors. However the peptide myr-FARKGALRQ reduced TSP-1 significantly to 61.5 ± 2.3% vs. control 100% ($P < 0.05$).

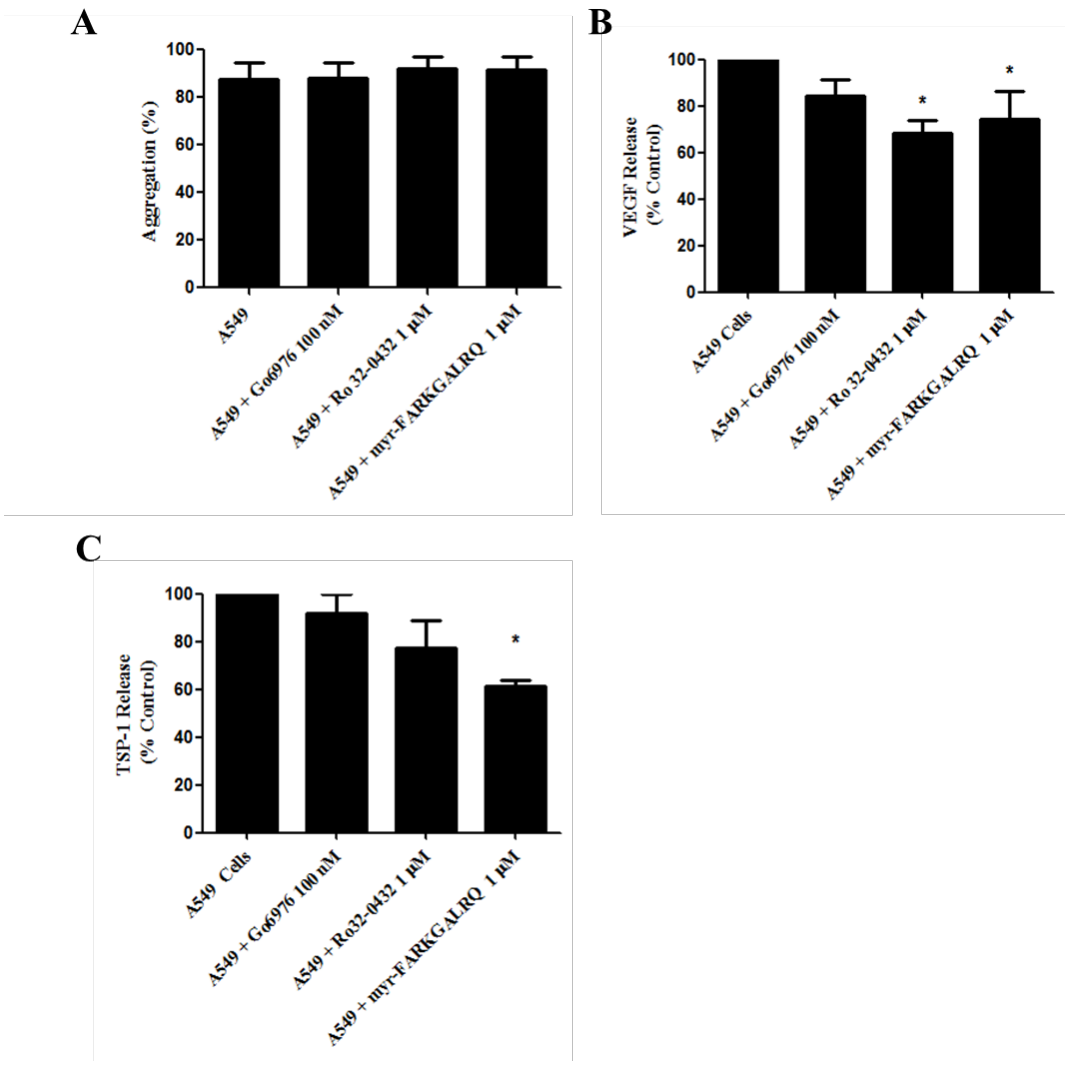


Figure 23: (A) Summary data of human platelets aggregated with A549 lung carcinoma cells in the presence of Gö 6976, Ro32-0432. Experiments demonstrating the inhibition in **(B)** $VEGF_{165}$ and **(C)** TSP-1 release from platelets aggregated with A549 cells by different concentrations of Gö 6976, Ro 32-0432 and myr-FARKGALRQ. N= 5, *, $P < 0.05$ vs. resting platelets (RP).

5. DISCUSSION

The fact that tumors are dependent on blood supply has led many researchers to search for anti-angiogenic molecules, and to design anti- angiogenic strategies for cancer treatment. Several approaches to block or disrupt tumor angiogenesis have been explored. Each step in the angiogenic regulatory pathway represents a potential target for therapeutic development. Perhaps the best validated anti-angiogenic approach involves blockade of the angiogenic signaling cascade, especially VEGF and its signaling pathway since approximately 60% of all cancers express VEGF (Folkman, 2006; Muñoz-Chápuli et al., 2004).

There are several approaches to inhibiting VEGF signalling including neutralization of the ligand or receptor by antibodies, and blocking VEGF receptor (VEGFR) activation and signalling with tyrosine kinase inhibitors (TKIs) selective for VEGFRs (Pieramici and Rabena, 2008). The US Food and Drug Administration approved the first generation anti-angiogenic drug, bevacizumab (Avastin) in 2004, which prevents VEGF binding to and activation of VEGFR1 and VEGFR2. Bevacizumab is used for treatment of colorectal cancer, non-small cell lung cancer and metastatic breast cancer in combination with chemotherapy. The TKI sorafenib has shown single-agent efficacy in patients with advanced renal cell carcinoma (RCC) and hepatocellular carcinoma (HCC) (Antoun et al., 2010). Sunitinib has also been shown to be efficacious as a single agent in patients with RCC (Ellis and Hicklin, 2008). It should be emphasized that, owing to their mode of action at the ATP binding pocket, TKIs are selective rather than specific for a particular kinase(s). Thus, TKIs designed to target VEGF receptors are actually considered ‘multi-kinase’ inhibitors having significant activity against Raf, PDGF receptor (PDGFRb) and FGF receptor (FGFR) (Bouïs et al., 2006; Mac Gabhann et al., 2010).

Indeed, inhibitors that block the VEGF pathway have shown to slow down the rapidly growing tumor vasculature, decreasing microvessel density and normalizing tumor blood vessels by decreasing permeability, interstitial pressure and tortuosity of the remaining blood vessels, thereby improving the delivery of

chemotherapy and augmenting radiation therapy (Folkman, 2007). However, despite the positive results seen with VEGF inhibitors, clinical resistance remains an issue with these drugs. Evasive resistance to VEGF pathway inhibitors include revascularization as a consequence to up-regulation of alternative pro-angiogenic signals; protection of the tumor vasculature either by recruitment of pro-angiogenic inflammatory cells or by increased protective pericyte coverage; accentuated invasiveness of tumor cells into local tissue with normal vasculature; and increased metastatic seeding and tumor cell growth in lymph nodes and distant organs (Bergers and Hanahan, 2008). The observed increased invasiveness and metastasis is in part due to the interaction of platelets with cancer cells (Sabrkhany et al., 2011; Cedervall and Olsson, 2010; Takashi et al., 2008).

Recently, it has been shown that a high platelet count is an independent adverse prognostic factor for overall survival of metastatic renal cell carcinoma patients treated with VEGF-targeted agents (Heng et al., 2009). As we have recently reviewed, numerous studies have shown that platelets of cancer patients have elevated levels of VEGF and other platelet-associated angiogenesis regulators (Radziwon-Balicka et al., 2012; Cervi et al., 2008; Peterson et al., 2012; Salgado et al., 1999; Wiesner et al., 2010; Caine et al., 2004). Since cancer patients also have highly reactive platelets (Jurasz et al., 2003b, 2004), VEGF and other platelet-associated angiogenesis regulators released from activated platelets of cancer patients may also contribute to the resistance of clinically used VEGF and VEGF pathway inhibitors. Therefore, additional novel targets and pathways need to be identified to broaden the anti-angiogenic drug spectrum and/or enhance the efficacy of current anti-angiogenesis drugs to overcome drug resistance. The inhibition of angiogenesis-regulator release from platelets may be such a novel target.

The fact that platelet granules, in particular α-granules contain a significant amount of proteins that regulate physiological and pathological processes such as angiogenesis, inflammation, immune response and tumor progression underlines their potential significance beyond hemostasis. Platelets store angiogenesis promoting growth factors in their α-granules and release them upon aggregation.

Several studies have identified conventional Protein Kinase C isoforms, PKCα and PKCβ as important signalling molecules regulating α-granule release (Konopatskaya et al., 2009; Pula et al., 2005b; Yoshioka et al., 2001) and platelet aggregation (Buensuceso et al., 2005; Giuliano et al., 2003), respectively. Hence, the goal of these studies was to investigate the effects of conventional PKC inhibitors, which have a lower IC_{50} for PKCα than PKCβ, on platelet aggregation and the release of angiogenesis regulating factors from α-granules. Titration of platelets with low concentrations of PKC inhibitors Gö 6976 (100 nM), myr-FARKGALRQ (1 μM), and Ro-32-0432 (1 μM) significantly inhibited α-granule release, as measured by P-selectin surface exposure and VEGF release. Importantly, Ro 32-0432 and myr-FARKGALRQ at this low concentration did not significantly inhibit platelet aggregation. The uncoupling of platelet secretory function from aggregate formation is not without precedent. A previous early study has shown that when platelet aggregation is suppressed by aspirin and a lack of external calcium, despite the inhibition of platelet aggregation, the PKC agonist 12-O-tetradecanoyl phorbol-12-acetate (TPA) can induce ATP secretion from platelet δ-granules (Rink et al., 1983). The current data demonstrates that by inhibiting PKC signaling the opposite uncoupling effect can be obtained, ie. suppression of granule secretion while maintaining aggregation.

Platelets have been shown to promote angiogenesis in wound healing via release of pro-angiogenic growth factors from platelet α-granules upon aggregation (Blair and Flaumenhaft, 2009). Angiogenesis is a process regulated by a very complex balance of stimulating growth factors, such as VEGF, PDGF and bFGF and inhibiting growth factors, such as angiostatin, endostatin and thrombospondin. Platelets contain both pro-angiogenic and anti-angiogenic molecules in their α-granules. Although platelets contain both pro-angiogenic and anti-angiogenic molecules, their overall effect is pro-angiogenic (Brill et al., 2004; Pinedo et al., 2012; Jurasz et al., 2003a). Reports in the literature have shown that the angiogenic molecules in α-granules can be differentially released (Ma et al., 2005; Italiano et al., 2008; Battinelli et al., 2011). Ma et al. were the first group to demonstrate that the release of pro-angiogenic and anti-angiogenic proteins by

platelets is a differentially regulated process that depends in part on stimulation of the thrombin receptors PAR-1 and PAR-4, which release VEGF or endostatin from platelet granules respectively. Thus, the signalling pathways that modulate the release of angiogenic molecules from platelets are complex (Ma et al., 2005). Subsequently, the Folkman group has shown that platelets store both pro-angiogenic and anti-angiogenic factors in separate subpopulations of α-granules and the content of these subpopulations can be differentially released by selective stimulation of PAR-1 and PAR-4 receptors (Italiano et al., 2008). Similar differential release from platelets was achieved via ADP-dependent signaling through the P2Y receptors (Bambace and Holmes, 2011), suggesting that multiple platelet signalling pathways may mediate differential release of angiogenesis regulating factors.

From these findings, it should not be suprising then if other signalling pathways such as those mediated by PKC isoforms also differentially release pro- vs. anti-angiogenesis regulating factors from platelets.. The current results show that Gö 6976, the most potent conventional PKC inhibitor tested, suppressed both VEGF and TSP-1 release from platelets. However, myr-FARKGALRQ (1 μM) and Ro-32-0432 (1 μM) both suppressed the release of the pro-angiogenic regulator vascular endothelial growth factor (VEGF) from α-granules but not the release of the anti-angiogenic molecule thrombospondin-1 (TSP-1). A possibility that explains the differential results achieved with conventional PKC inhibitors is that other PKCs besides PKCα may be involved in regulating release of α-granules (Chari et al., 2009b) containing TSP-1, and the more potent Gö 6976 also inhibits the other possible PKCs indirectly.

Human platelets express different protein kinase C (PKC) isoforms, namely α, $\beta_{I/II,}$ θ, ε, δ, η, and ζ, which phosphorylate multiple proteins during platelet activation which suggest different functions for each isoform (Heemskerk et al., 2011; Harper and Poole, 2010). PKC has been shown to have a role in granule secretion in both Ca^{2+}-dependent and Ca^{2+}-independent granule secretion (Rink et al., 1983; Yoshioka et al., 2001; Flaumenhaft, 2003). The use of PKC inhibitors Gö 6976, GF109203X, U73122, have been shown to block granule release in the

past (Pula et al., 2005a; Toullec et al., 1991; Watanabe, 2001); however, the advent of more selective inhibitors and the use of knockout animal models have elucidated specific roles of the different PKC isoforms in granule release. In this regard, studies using mice lacking PKCα have demonstrated key roles of PKCα in the release of both α-granules and dense granules in thrombin and collagen stimulated platelets (Konopatskaya et al., 2009). The platelets of PKCα null mice used to demonstrate the importance of this kinase for α-granule release upon aggregation also have the defect that they lack δ-granules and the ADP normally stored within them to mediate aggregation (Konopatskaya et al., 2009). This defect exposes the difficulty that the platelets studied by mouse models are not similar to the physiological conditions present in human platelets.

The conventional isoform PKCβ has been shown to positively regulate outside-in signaling by integrin αIIbβ3 in mouse platelets, a process that is involved in the overall changes undergone by the cytoskeleton in platelet aggregation and platelet spreading (Buensuceso et al., 2005). The current studies are the first to demonstrate that low concentrations of some conventional PKC inhibitors can inhibit the release of α- and dense-granules with minimal effects on platelet aggregation. The current results are also in line with Flaumenhaft and colleagues who have recently shown that pharmacological inhibition of platelet actin polymerization abolishes α-granule secretion but not aggregation (Woronowicz et al., 2010).

The concentrations of Gö 6976 and myr-FARKGALRQ used for this study were lower than those used in other studies involving human platelets (Harper and Poole, 2010; Gilio et al., 2010a). The lower concentration of Gö 6976 and Ro 32-0432 (which has not been previously studied) was specifically used to increase the isoform selectivity of the inhibitor (preferentially target PKCα over β). The pseudosubstrate peptide inhibitor myr-FARKGALRQ was used at low concentrations so as not to inhibit a previously reported ecto-protein kinase C on the platelet surface membrane that maintains the latency of fibrinogen receptors and hence whose inhibition enhances aggregation (Babinska et al., 1996, 2000). While at maximally effective concentrations of the inhibitors used granule release

and platelet aggregation were abolished, submaximal concentrations led to different degrees of inhibition of each process. The limitation of using pharmacological inhibitors is that singular targeting of PKCα vs. PKCβ cannot be totally guaranteed although the different IC_{50} values of Gö 6976 and Ro 32-0432 would appear to favor mainly PKCα inhibition. Although in many aspects human platelets are similar to mouse platelets, they also present clear differences (Ahrens and Peter, 2008). Mouse platelets are smaller than human platelets and differ in the densities of various receptors (Ware et al., 1993). The platelet count is higher in mice than in humans. Mouse and human platelets also differ in their receptor repertoire (e.g., protease-activated receptors (PAR), FcgRIIa) (Connolly et al., 1996). Thus, conventional mouse thrombosis models may not be suitable to be the only tool used to study and develop drugs that target specific human signaling pathways.

PKC directly phosphorylates multiple proteins involved in secretion including SNARE proteins and their chaperones, SNAP-23, syntaxin 4, syntaxin 2, Munc13c and Munc18c (Ren et al., 2010; Houng et al., 2003; Chung et al., 2000; Flaumenhaft et al., 2007; Polgár et al., 2003b). PKC also phosphorylates the proteins associated with the cytoskeleton pleckstrin and MARCKS (Elzagallaai et al., 2012; Lian et al., 2009) and other kinases such as type II phosphatidylinositol-5-phosphate-4-kinase (PIPK) and protein kinase D (PKD) (Rozenvayn and Flaumenhaft, 2003; Konopatskaya et al., 2011). The differential effects on VEGF and TSP-1 release in our experiments by the use of inhibitors of conventional PKCs could be due to impaired phosphorylation of downstream targets that occurs in a concentration-response manner.

Thrombin is a very potent agonist for platelet activation (Keuren et al., 2005). Thrombin has been also shown to contribute to a more malignant phenotype by activating platelet-tumor aggregation, increasing tumor adhesion to subendothelial matrix and tumor-associated angiogenesis (Nierodzik and Karpatkin, 2006). Our results indicate that the concentration-dependent inhibition by cPKC inhibitors of platelet aggregation and granule secretion is abscent when thrombin is used to activate the platelets. These results suggest that conventional PKCs act within a

collagen-dependent pathway for α-granule release and aggregation. Other studies involving mouse platelets have shown that stimulation of the PAR-1 and PAR-4 receptors with selective agonists have a differential effect on the release of α-granule contents (Ma et al., 2005; Italiano et al., 2008). Hence, it was surprising that with cPKC inhibition of thrombin-activated platelets did not result in inhibition of α-granule release. However, is important to mention that thrombin induces actin polymerization in a tyrosine kinase-dependent and Ca^{2+}-independent manner (Rosado et al., 2000); therefore, offering a likely alternative to the PKCα signaling associated with collagen-stimulated α-granule release. Furthermore, PKC inhibition has been shown to enhance thrombin-induced Ca^{2+} signaling likely maintaining aggregation in the presence of PKC inhibition (Harper and Poole, 2011).

Experiments using confocal immunofluorescence microscopy revealed that inhibition with Ro-32-0432 (1 μM) prevented the translocation of PKCα to the α-granule, with no apparent effects on PKCβ translocation. These results suggest that the subcellular location of the activated PKC defines the signaling pathway and selective role played by that conventional PKC isoform. The current study suggests a direct link between translocation and PKC isoform specific functions in platelets. Platelet PKC translocation is likely an area of research that should be further investigated in the future.

Even though the current data showed reduced release of angiogenesis regulators from platelets incubated with low concentrations of conventional PKC inhibitors, it was important to determine if this reduced release had any functional effects on angiogenesis; hence, *in vitro* angiogenic assays were performed. A decrease in the area covered by capillary-like tubes formed by human microvascular endothelial cells (HMVEC) was observed in the presence of releasates from platelets inhibited with Ro-32-0432 (1 μM) but not Gö 6976 (100 nM). These differential results may be explained by the capability of Ro 32-0432 to lower the levels of VEGF released by platelets to a greater extent than TSP-1, and thus perhaps modifying the overall platelet-angiogenic balance (Hanahan and Folkman, 1996). Our observations are in accordance to previously reported effects

of platelet blockade with aspirin on suppressing angiogenesis (Battinelli et al., 2011). The ability of conventional PKC inhibitors to impair platelet-stimulated angiogenesis could potentially be translated into settings of pathological angiogenesis such as tumour angiogenesis, since growth factors released from platelets can be utilized by growing tumours makeing platelets an interesting target for anti-angiogenic therapy (Browder et al., 2000).

A high platelet count has been shown to indicate a poor outcome in many cancers (Jain et al., 2010b; Dineen et al., 2009; Bambace and Holmes, 2011). This is likely a result of the tumor angiogenesis promoting effects of platelets. Low platelet counts often occur in patients undergoing myelosuppressive chemotherapy due to impaired formation of new platelets. Cancer patients with low platelet numbers are at risk of bleeding since platelets are responsible for plug formation. Therefore, these patients receive platelet transfusions. Unfortunately, platelet transfusion replenishes the growth factors found in platelets that support blood vessel growth to tumors. Thus, prior to transfusion to the cancer patient, it would be beneficial to prevent blood vessel growth factor release from platelets to inhibit tumor growth, but at the same time maintain clot formation to prevent bleeding. Selective platelet PKCα inhibition may be of benefit to reduce the effects of platelet-stimulated angiogenesis in the cancer setting, while still maintaining plug formation capabilities of platelets.

Many studies have shown that tumor cells have the ability to induce platelet aggregation and that this is correlated positively with their metastatic potential *in vivo* (Jurasz et al., 2003b, 2004; Menter et al., 1987; Gasic, 1968; Radomski et al., 1980). Platelets also are involved in tumour malignancy by helping tumour cells evade the body's immune systems, offering protection from high shear forces seen in flowing blood and preventing intra-tumor hemorrhage (Jurasz et al., 2004; Sabrkhany et al., 2011; Kisucka et al., 2006). Hence, the effects of conventional PKC inhibition on tumor cell-induced platelet aggregation (TCIPA) and angiogenesis regulator release were investigated. Significant reduction of VEGF was observed with both Ro 32-0432 (1μM) and myr-FARKGALRQ (1 μM). TSP-1 release was only reduced by the peptide inhibitor myr-FARKGALRQ (1 μM).

However none of the cPKC inhibitors affected aggregation which would suggest that TCIPA acts through different pathways than angiogenesis regulator release that not involves cPKC signaling.

6. CONCLUDING REMARKS

Pharmacological inhibition of conventional PKC isoforms PKCα and PKCβ differentially regulate collagen-mediated platelet functional responses that include platelet aggregation, dense and α-granule secretion. Therefore, the current studies identify molecular mechanisms underlying differential regulation of platelet functions. In summary the results indicate that:

1) Careful titration of PKC inhibitors preferentially prevents the release of the pro-angiogenic factor VEGF over the anti-angiogenic factor TSP-1 from platelet α-granules.

2) Inhibition of VEGF release from α-granules may occur independent of platelet aggregation

3) Inhibiting VEGF release from platelets has functional consequences inhibiting platelet-stimulated angiogenesis.

4) Conventional PKC inhibition at submaximal concentrations prevents translocation of PKCα to platelet granules.

The most potent inhibitor identified in these studies for the uncoupling of aggregation and granule release was Gö 6976. These studies have identified PKCα as a suitable target for the development of novel and easier to titrate compounds that prevent the release of angiogenesis promoters from platelets. In theory novel compounds that selectively inhibit PKCα could be used to treat platelets prior to their transfusion into thrombocytopenic cancer patients to prevent the release of angiogenesis promoters without altering their aggregating capabilities.

7. FUTURE DIRECTIONS

These studies have shown that conventional PKCs, in particular PKCα is important for α-granule release of pro-angiogenic regulators like VEGF. These conclusions were reached based on the use of pharmacological inhibitors. The use of inhibitors always presents the limitation of specificity, which is why it would be important to corroborate these results with other approaches, such as knockout mice models. In the future PKCα and PKCβ deficient mice, although not perfect models themselves, would offer new insights on the effects of the two PKC isoforms on angiogenesis regulator release from platelets stimulated with different agonist.

Further investigations on the possible involvement of other PKC isoforms (Chari et al., 2009c, 2009b) and other kinases like PKD (Konopatskaya et al., 2011) on platelet activation pathways involving platelet stimulation by thrombin would expand our knowledge on the signaling pathways involved in different platelet processes and offer new potential clinical targets. The fact that PKC inhibition failed to inhibit thrombin-induced α-granule release suggests different signalling mechanisms occurring downstream of PAR and collagen (GPVI and $\alpha_2\beta_1$) receptors in human platelets. Use of transgenic mice models and thrombin receptor antagonists will be helpful in elucidating the different mechanisms by which collagen and thrombin stimulate platelet angiogenesis regulator release.

Functional effects of conventional platelet PKC inhibition on angiogenesis still needs to be studied to be investigated in an *in vivo* angiogenesis model. Experiments using a matrigel plug assay or chick chorioallantoic membrane (CAM) angiogenesis assays may provide valuable information to complement the findings of the current work.

Finally, a potential future research direction is the investigation of potential links between pharmacological PKC inhibition and any of the SNARE

molecules involved in the α-granule release machinery. Since PKCα is important in the release of the pro-angiogenic subpopulation of α-granules, then it is likely that it directly or indirectly interact with subsets of SNARE molecules, like syntaxin-2 or syntaxin-4, in a differential manner (Ren et al., 2008).

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