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Pharmacologic Protein Kinase Cα Inhibition Uncouples Human Platelet-Stimulated Angiogenesis from Collagen-Induced Aggregation[S]

Cesar Moncada de la Rosa, Aneta Radziwon-Balicka, Haitham El-Sikhry, John Seubert, Peter P. Ruvolo, Marek W. Radomski, and Paul Jurasz

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (C.M.R., A.R.B., H.E.-S., J.S., P.J.), and Department of Pharmacology (J.S., P.J.) and Cardiovascular Research Centre (C.M.R., A.R.B., H.E.-S., J.S., P.J.), Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta, Canada; Department of Leukemia Research, University of Texas, Maryland Anderson Cancer Center, Houston, Texas (P.P.R.); School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin, Ireland (M.W.R.)

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ABSTRACT

Platelets promote angiogenesis by releasing angiogenesis-regulating factors from their α-granules upon aggregation. This effect has both physiologic and pathologic significance as it may contribute to carcinogenesis. Platelet α-granule release and aggregation are regulated, in part, via protein kinase C (PKC) α and β signaling. Our study investigated the effects of PKC inhibition on aggregation, angiogenesis-regulator secretion from α-granules, and platelet-stimulated angiogenesis. We hypothesized that selective PKCα inhibition may preferentially suppress angiogenesis-regulator secretion from α-granules but not aggregation, limiting platelet-stimulated angiogenesis. Human platelets were aggregated in the presence of conventional PKC inhibitors myr-FARKGALRQ and Ro 32-0432 (2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyridol[1,2-α]indol-3-yl}-3-(1-methyl-1*H*-indol-3-yl)maleimide). Immunofluorescence microscopy of PKC translocation was used to determine the specificity of PKC-inhibitor targeting. Enzyme-linked immunosorbent assay was used to measure vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1) release from platelets. Platelet effects on angiogenesis were tested using a capillary-formation assay. Ro 32-0432, but not the peptide inhibitor myr-FARKGALRQ (myristoylated-pseudosubstrate peptide inhibitor), inhibited aggregation in a concentration-dependent manner, while both Ro 32-0432 and myr-FARKGALRQ preferentially suppressed VEGF over TSP-1 secretion. Suppression of angiogenesis-regulator release occurred at inhibitor concentrations that did not significantly affect aggregation. Immunofluorescence microscopy revealed that PKCα targeting to α-granules is inhibited when angiogenesis-regulator secretion is uncoupled from aggregation. At concentrations that uncoupled α-granule release from aggregation, Ro 32-0432 and myr-FARKGALRQ inhibited platelet-stimulated angiogenesis. Hence, selective PKCα inhibition suppresses angiogenesis-regulator release from platelet α-granules with minimal effects on aggregation. Thus, selective PKCα inhibitors may have pharmacologic significance to regulate platelet-promoted angiogenesis.

Introduction

In addition to maintaining hemostasis, platelets are one of the largest circulating reservoirs of angiogenesis regulators which when released from platelets have the potential to significantly promote angiogenesis (Ma et al., 2001; Jurasz et al., 2003; Radziwon-Balicka et al., 2012a). Platelets store and release upon aggregation angiogenesis promoters such as vascular endothelial growth factor A (VEGF-A) (Mohle et al., 1997), platelet-derived growth factor (Bar et al., 1989), basic fibroblastic growth factor (bFGF) (Kaplan et al., 1979), epidermal growth factor (Pesonen et al., 1989), angiopoietin (Li et al., 2001), and matrix metalloproteinases (Sawicki et al., 1997). To counterbalance the angiogenesis promoters, platelets also store and release upon aggregation angiogenesis inhibitors including thrombospondin (TSP-1) (Zaslavsky et al., 2010), platelet factor-4 (Maione et al., 1990), endostatin (Ma et al., 2005), tissue inhibitors of matrix metalloproteinases (Radomski et al., 2002), and angiostatin (Jurasz et al., 2003). The majority of these platelet-associated angiogenesis regulators are found in separate populations of platelet α-granules

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ABBREVIATIONS: bFGF, basic fibroblastic growth factor; ELISA, enzyme-linked immunosorbent assay; GRK, G-protein-coupled receptor kinase; HMVEC-L, human microvascular endothelial cell from lung; myr-FARKGALRQ, myristoylated-pseudosubstrate peptide inhibitor; PBS, phosphate-buffered saline; PKC, protein kinase C; PKCβ-inhibitor, 3-(1-(3-imidazol-1-ylpropyl)-1*H*-indol-3-yl)-4-anilino-1*H*-pyrrole-2,5-dione; PMA, phorbol myristate acetate; PRP, platelet rich plasma; Ro 32-0432, 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyridol[1,2-α]indol-3-yl}-3-(1-methyl-1*H*-indol-3-yl)maleimide; rottlerin, (E)-1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethylchromen-8-yl]-3-phenylprop-2-en-1-one; TSP-1, thrombospondin-1; VEGF, vascular endothelial growth factor.

that can be differentially released upon aggregation (Ma et al., 2005; Italiano et al., 2008).

Platelet aggregation and α-granule releases are regulated, in part, by protein kinase C (PKC) signaling (Yoshioka et al., 2001; Tabuchi et al., 2003; Chari et al., 2009; Konopatskaya et al., 2009; Gilio et al., 2010). The PKC isoenzymes are a family of serine/threonine protein kinases that are subdivided into three classes, including the conventional (α, βI, βII, γ), novel (δ, ε, η, θ), and atypical (λ,ι, μ,ξ) PKCs. The conventional PKC isoenzymes, which in human platelets include PKCα and β (Harper and Poole, 2010), have been shown to contribute to the intracellular signaling that mediates α-granule release and aggregation (Yoshioka et al., 2001; Tabuchi et al., 2003; Konopatskaya et al., 2009; Gilio et al., 2010). Although, a certain redundancy in activator signaling by PKCα and β may exist in human platelets, studies have shown PKCα to be essential for α-granule release, whereas PKCβ may be more important in regulating $\alpha_{IIb}\beta_3$ signaling during aggregation (Yoshioka et al., 2001; Buensuceso et al., 2005; Gilio et al., 2010). PKCα has been shown to be essential for α-granule release by permeabilized human platelets (Yoshioka et al., 2001), and PKCα null mice have substantially impaired release of α-granule contents upon aggregation (Konopatskaya et al., 2009). Although PKCα null mice also have a defect in platelet aggregation and thrombus formation, they also have reduced numbers of δ-granules and potentially the ADP normally stored within them to mediate aggregation. PKCβ on the other hand has been found to immunoprecipitate with platelet integrin αIIb/β3, while PKCβ null mice display impaired spreading on fibrinogen (Buensuceso et al., 2005). Similarly, pharmacologic studies have shown that PKC inhibition by small molecule inhibitors, particularly those designed to inhibit the conventional class, can inhibit platelet aggregation and α-granule release (Pula et al., 2005; Gilio et al., 2010).

However, the impact of PKC inhibition on angiogenesis regulator release during aggregation or platelet-stimulated angiogenesis has not been previously investigated. Studies using common antiplatelet agents such as aspirin and ticlopidine have demonstrated that, in addition to impairing platelet hemostatic function, these antiplatelet agents also impair angiogenesis regulator release from platelets and platelet-induced angiogenesis (Ma et al., 2001; Battinelli et al., 2011). Hence, we hypothesized that, similar to antiplatelet agents, platelet PKC inhibition would suppress angiogenesis regulator release from platelet α-granules and platelet-stimulated angiogenesis. Moreover, if PKCα and β have distinct functional roles in regulating α-granule release and aggregation respectively, we further hypothesized that PKCα may preferentially regulate platelet-stimulated angiogenesis.

Materials and Methods

Reagents. Cell-permeable myristoylated-pseudosubstrate peptide inhibitor myr-FARKGALRQ, Ro 32-0432 (2-{8-[(dimethylamino) methyl]-6,7,8,9-tetrahydropyridol[1,2-α]indol-3-yl}-3-(1-methyl-1*H*-indol-3-yl)maleimide), PKCβ-inhibitor [3-(1-[3-imidazol-1-ylpropyl]-1*H*-indol-3-yl)-4-anilino-1*H*-pyrrole-2,5-dione], and rottlerin [(E)-1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethylchromen-8-yl]-3-phenylprop-2-en-1-one] were obtained from Calbiochem (Mississauga, ON, Canada). Collagen, thrombin, and Chrono-Lume reagent were obtained from Chronolog (Havertown, PA). Bovine serum albumin and phorbol myristate acetate (PMA) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Matrigel, allophycocyanin-labeled anti-human-CD62P (P-selectin) (clone AK4), and fluorescein isothiocyanate-labeled PAC-1 monoclonal antibodies were purchased from BD Biosciences (Mississauga, ON, Canada). Antibodies for PKCα (clone Y143), phospho-T497-PKCα (clone EP2608Y), PKCβI/βII C-terminal, and phospho-S660-PKCβII (clone EP1902Y) were obtained from Abcam (Cambridge, MA). An anti-bFGF antibody was obtained from R&D Systems (Minneapolis, MN). Unlabeled anti-human-CD62P (clone AK4) was obtained from BD Biosciences. Anti-Rabbit Dy-Light 548 and Anti-Mouse Dy-Light 488 were obtained from Jackson ImmunoResearch, Inc. (West Grove, PA).

Blood Platelets and Platelet Aggregation. Approval for the current study was obtained from the University of Alberta Human Research Ethics Board. Informed consent was provided by and blood collected from healthy volunteers who had not taken any drugs known to affect platelet function for 14 days before the study. Prostacyclin-washed platelet suspensions were prepared as previously described elsewhere (Jurasz et al., 2001a, 2003). Briefly, prostacyclin (0.06 μg/ml) was added to whole blood followed by centrifugation at 250*g* for 20 minutes to isolate platelet rich plasma (PRP). Next, prostacyclin (0.3 μg/ml) was added to PRP, and platelets were pelleted at 900*g* for 10 minutes. The platelet pellet was subsequently washed 3 times with Tyrode's buffer and resuspended at 2.5×10^8/ml. Washed platelets were allowed to rest at room temperature for 1 hour for the platelet inhibitory effects of prostacyclin to wear off. Washed platelets were preincubated for 2–10 minutes at 37°C (900*g*) in a whole-blood ionized calcium lumi-aggregometer (Chronolog) with PKC inhibitors or vehicle. Platelet aggregation was then initiated by the addition of collagen (10 μg/ml) and monitored by Aggro-Link software for up to 15 minutes as previously described elsewhere (Jurasz et al., 2001a,b). After aggregation, platelet pellets were separated from releasates using centrifugation (1000*g* for 10 minutes) and then stored at −80°C for analysis of angiogenesis regulator release.

Alternatively, for matrigel experiments in which washed-platelet releasates were needed to be devoid of PKC inhibitors, isolated PRP was incubated with conventional PKC inhibitors (10 minutes). Then platelets were pelleted at 900*g* for 10 minutes and resuspended in Tyrode's buffer (10 ml). Platelets were washed in this manner 3 times to remove free inhibitors from platelet suspensions prior to aggregation. Washed platelet suspensions were then prepared as previously described, 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 angiogenesis assays.

Flow Cytometry. Flow cytometry was performed using Beckman Coulter FC500 or Quanta SC flow cytometers (Beckman Coulter, Mississauga, ON, Canada) on single stained platelet samples as described previously elsewhere (Jurasz et al., 2001b). Briefly, to minimize the presence of large aggregates in samples, platelets (10 μl of suspension) were taken from the aggregometer at 50% light transmittance, or equivalent time point, and added to fluorescent-labeled antibodies (10 μl) containing 0.25 μg of anti-P-selectin then diluted 10-fold using physiologic saline. Samples and antibodies were incubated in the dark at room temperature for 5 minutes. Platelets were identified by forward (or electronic volume) and side scatter signals, and 10,000 platelet-specific events were analyzed by the cytometer for mean fluorescence.

Measurement of ATP Release by Chemiluminescence. ATP release from platelet dense granules by a whole blood ionized calcium lumi-aggregometer was performed as previously described elsewhere (Chung et al., 2002). Briefly, platelets were incubated with luciferin-luciferase reagent (440 luciferase units/ml and 4 mg/ml of luciferin) for 10 minutes at 37°C in the presence of PKC inhibitors. After incubation, collagen (10 μg/ml) was added, and the luminescence was monitored. To quantify the release of platelet ATP, standard curves were constructed with ATP standard (Chronolog). The data were expressed as nM ATP or in some experiments as percentage of maximal release.

Western Blot Analysis. Western blot analysis was performed as described previously elsewhere (Jurasz et al., 2003). Briefly, platelet pellets were lysed (10–45 μg protein per lane) and subjected to 8 or 12% SDS-PAGE. After electrophoresis and transfer of samples onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA), the blots were blocked overnight in blocking buffer and then incubated with the primary antibodies against PKCα (1:2000), phospho-T497-PKCα (clone EP2608Y) (1:5000), PKCβI/βII C-terminal (1:5000), phospho-S660-PKCβII (1:10,000), or bFGF (0.2 μg/ml) for 2 hours. Anti-rabbit (1:5000) or anti-goat (0.8 μg/ml) horseradish peroxidase–labeled antibodies were used as the secondary antibody (Sigma-Aldrich). The immunoreactive bands were visualized with an ECL Plus kit (Amersham Biosciences, San Francisco, CA). In some instances, membranes were stripped and probed for β-actin with a β-actin-horseradish peroxidase–conjugated antibody (1:25,000) (Sigma-Aldrich), which was used as a loading control. Chemiluminescence was detected using a VersaDoc MP5000 molecular imager with Quantity One software (Bio-Rad).

Enzyme-Linked Immunosorbent Assay. $VEGF_{165}$ and TSP-1 released by platelets were quantified by enzyme-linked immunosorbent assay (ELISA). The ELISAs were performed on releasates from unactivated and collagen-aggregated platelets as per manufacturer's instructions and described previously elsewhere (Jurasz et al., 2011). The baseline passive release of VEGF and TSP-1 by unactivated platelets that were stirred (900 rpm) in the lumi-aggregometer (for an equivalent time to that of aggregation samples) was subtracted from that of collagen-aggregated platelets, and the release results were expressed as the percentage of control collagen-aggregated platelets.

Cell Culture. Human microvascular endothelial cells derived from lung (HMVEC-L) were obtained from Lonza (Walkerville, MD). HMVEC-L were cultured in Endothelial Cell Growth Medium 2-MV (Lonza), as described previously elsewhere (Jurasz et al., 2011).

In Vitro Angiogenesis Assays. In vitro angiogenesis assays in which endothelial cells align and form hollow tube capillary-like structures on Matrigel were performed as described previously elsewhere (Jurasz et al., 2003, 2006) with the following modifications. Briefly, Matrigel was mixed with platelet releasates (1:3) on ice and allowed to solidify at 37°C for 30 minutes in 96-well plates. Subsequently, 1×10^4 HMVEC-L were added in Endothelial Basal Medium-2 (Lonza) to each well and incubated up to 48 hours at 37°C in a humidified atmosphere with 5% CO_2. At 6-, 12-, 24-, and 48-hour time points, the capillary-like structures formed by HMVEC-L were documented using a Olympus CKX41 microscope (Olympus America Inc., Melville, NY) equipped with a digital camera. Photomicrographs were converted to binary (skeletonized) images, and the areas covered by capillary-like structures and the number of branch points were determined using Image J software (NIH, Bethesda, MD; http://rsbweb.nih.gov/ij/).

Migration Assays. Endothelial cell migration assays were performed as described previously elsewhere (Radziwon-Balicka et al., 2012b) with the following modifications. We added 3×10^5 HMVEC-L to each gelatin-coated insert of a 6-well plate and allowed them to migrate toward a gradient formed by adding isolated platelet releasates (2 ml) to the wells of insert companion plates. After 24 hours, the nonmigrating HMVEC-L were removed by scrubbing the upper surface of the inserts with a cotton-tipped swab. The cells on the lower surface membrane were fixed in 4% formaldehyde in phosphate-buffered saline (PBS), stained with Diff-Quik stain, then examined by light microscopy.

Platelet Transmission Electron Microscopy and Dual-Immunofluorescence Confocal Microscopy. Transmission electron microscopy was performed as described previously elsewhere (Radomski et al., 2005). Briefly, platelet ultrathin sections were cut using an LKB Ultracut microtome (Leica, Deerfield, IL) then stained with uranyl acetate and lead citrate in an LKB Ultrostainer. Transmission electron microscopy was performed using a JEM 1010 transmission electron microscope (JEOL Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using the AMT Advantage digital CCD camera system (Advanced Microscopy Techniques Corp., Danvers, MA)

For immunofluorescence microscopy, platelets were fixed for 20 minutes in 4% formaldehyde in Tyrode's buffer. Solutions of fixed platelets were cytospinned onto polylysine-coated coverslips at 250*g* for 5 minutes. Platelets were permeabilized with Tyrode's buffer containing 0.1% Triton X-100. Specimens were blocked overnight in PBS with 5% bovine serum albumin, followed by incubation with anti-PKCα or anti-PKCβI/βII C-terminal antibodies (1:50 dilution) for 2 hours. Coverslips were washed 3 times with PBS, treated with appropriate secondary antibody for 2 hours, then washed 3 times with PBS. Subsequently, coverslips were incubated with anti-CD62P antibody (1:100 dilution) in the manner described earlier. Controls were treated in the same fashion except for exclusion of the primary antibody. Preparations were mounted in Prolong Gold Antifade solution (Invitrogen, Carlsbad, CA) 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 milliseconds.

Statistical Analysis. For the analysis of variances between groups of data, one-way analysis of variance was performed followed by Dunnett test using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA), and paired *t* tests also were performed where appropriate. Data are expressed as mean ± S.E.M. $P < 0.05$ was considered statistically significant.

Results

Conventional Isoform PKC Inhibitors Inhibit Platelet Aggregation More Potently Than α-Granule Release. A cell-permeable myristoylated pseudosubstrate peptide inhibitor myr-FARKGALRQ specific for PKCα and β (Kubo et al., 1987; Eichholtz et al., 1993) did not inhibit platelet aggregation in the 0–1 μM range, but inhibited P-selectin surface exposure (100.0% ± 0.0% versus 32.2% ± 22.8%, $P < 0.05$) (Fig. 1, A–D). This uncoupling of platelet aggregation from α-granule release was also achieved with the conventional PKC inhibitor Ro 32-0432 (cell free PKCα IC50 9 nM versus PKCβ IC50 28 nM) (Fig. 1, E and F). At 1 μM, Ro 32-0432 did not significantly inhibit platelet aggregation compared with control (78.6% ± 1.0% versus 68.2% ± 4.7% light transmittance, $P > 0.05$) but did inhibit P-selectin surface exposure (100.0% ± 0.0% versus 55.9% ± 15.8%, $P < 0.05$). In contrast, the uncoupling of platelet aggregation from α-granule release was not achieved by the nitric oxide donor s-nitroso-glutathione (GSNO) (Fig. 2, A and B) nor by an aniline-monoindolylmaleimide PKCβ inhibitor (PKCβII IC50 5 nM and PKCβI IC50 21 nM versus PKCα IC50 331 nM) (Fig. 2, C and D).

Conventional PKC Inhibitors Interfere with Platelet PKCα Targeting. Examination of resting platelets by electron microscopy demonstrates granules scattered throughout the platelet, but upon aggregation the platelets concentrate their granules at their centers (centralize) for release (Supplemental Fig. 1A). To determine whether conventional PKC inhibitors at concentrations that 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 "targeting" (a process dependent on catalytic competence) to these centralized granules after activation by collagen.

We first confirmed that collagen activated both PKCα and β by detecting their phosphorylated forms by immunoblot analysis (Supplemental Fig. 2). The phospho-PKC specific bands also corresponded to bands measuring total PKCα and β, thus confirming the specificity of these antibodies for confocal immunofluorescence microscopy. Within resting platelets, PKCα was primarily found to localize to the plasma membrane (Fig.

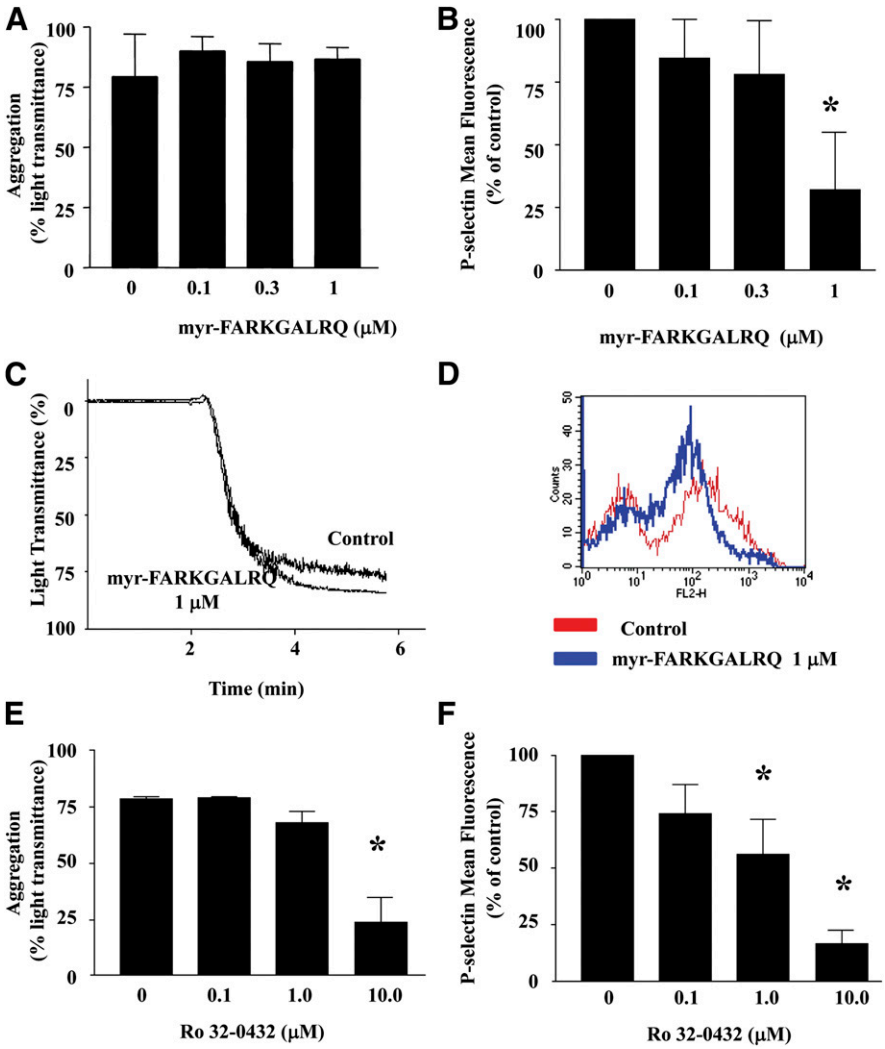

Fig. 1. Conventional PKC inhibitors uncouple α-granule release from aggregation, as shown in the summary data and representative experiments of collagen (10 μg/ml) induced platelet aggregation and P-selectin surface expression in the presence of myr-FARKGALRQ (A–D) and Ro 32-0432 (E and F). Platelet P-selectin surface exposure was measured by flow cytometry. $N = 4$ experiments. *$P <$ 0.05 versus control.

3A*i*), while the PKCβ isoform was found around the plasma membrane and throughout the platelet cytosol (Fig. 3B*i*). P-selectin immunostaining was punctuate consistent with its localization within α-granules. Upon activation by collagen, PKCα translocated to the platelet center and colocalized with P-selectin in individual activated platelets, indicating its targeting to the α-granule (Supplemental Fig. 1B). Within aggregates in which individual platelets could still be distinguished, P-selectin released from α-granules translocated to the platelet surface membrane while PKCα remained within platelet centers (Fig. 3A*ii*). PKCβ did not translocate to the centers of platelets upon activation by collagen (Fig. 3B*ii*; Supplemental Fig. 1C). 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 unreleased from the central granular cores of many platelets (Fig. 3A*iii*). Ro 32-0432 (1 μM) exerted no apparent effect on PKCβ targeting (Fig. 3B*iii*). 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 (Fig. 3C).

Uncoupling of Platelet Aggregation from α-Granule Release via PKC Inhibition Suppresses Angiogenesis Regulator Release from Platelets. To determine the consequences of uncoupling platelet aggregation from α-granule release on angiogenesis regulator release, we used ELISA to measure the VEGF and TSP-1 released after aggregation. Depending on donor platelets, 50–200 pg/ml of VEGF was released from α-granules when stimulated by 10 μg/ml collagen. The small molecule inhibitor Ro 32-0432 inhibited platelet VEGF release starting at 1 μM (100.0% $\pm$ 0.0% versus 47.3% $\pm$ 23.6%, $P < 0.05$), a concentration that did not significantly inhibit aggregation (Fig. 4A). Although Ro 32-0432 failed to suppress TSP-1 release at 1 μM, it did so at 10 μM (Fig. 4B). Interestingly, the cell-permeable myristoylated pseudosubstrate peptide inhibitor myr-FARKGALRQ (1 μM) also inhibited VEGF but not TSP-1 release in response to collagen (Fig. 4, C and D), indicating preferential inhibition of α-granules or α-granule compartments storing a proangiogenic mediator. This inhibition in the release of a proangiogenic α-granule mediator by myr-FARKGALRQ (1 μM) but not platelet aggregation was further confirmed by bFGF immunoblot analysis of collagen-aggregated platelet lysates (Supplemental Fig. 3).

To investigate whether other isoform PKC inhibitors would be able to uncouple collagen-induced platelet aggregation from angiogenesis regulator release from α-granules, we tested the effects of the putative PKCδ inhibitor rottlerin on collagen-stimulated aggregation and angiogenesis regulator release. Interestingly, rottlerin at 10 μM significantly inhibited platelet aggregation but did not prevent VEGF or TSP-1 release from platelets, an opposite effect to that of the

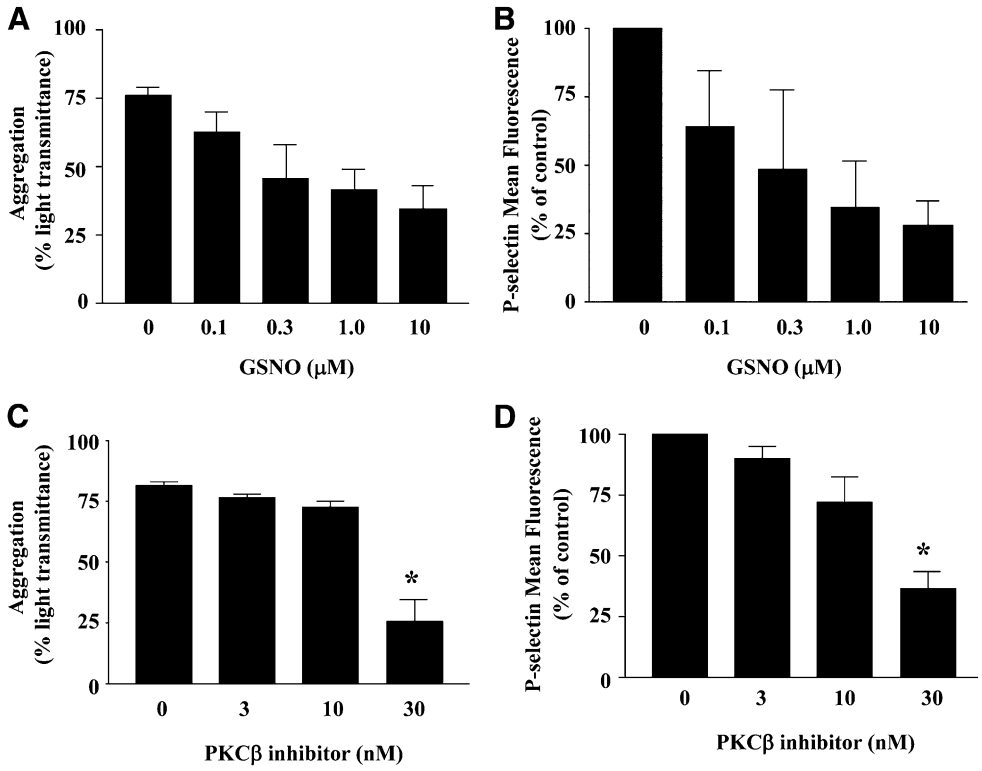

Fig. 2. Summary data show collagen (10 μg/ml)-induced platelet aggregation and P-selectin surface expression in the presence of GSNO (A and B) and PKCβ inhibitor (C and D). Platelet P-selectin surface exposure was measured by flow cytometry. N = 4 and 5 experiments, respectively. *$P < 0.05$ versus control.

conventional PKC inhibitors (Supplemental Fig. 4, A–C). However, at a high concentration (30 μM) that almost completely blocked platelet aggregation, rottlerin significantly inhibited VEGF but not TSP-1 release, similar to myr-FARKGALRQ (Supplemental Fig. 4, B and C).

Inhibition of PKC-Dependent α-Granule Release Ameliorates Capillary Tube Formation on Matrigel. To determine whether inhibition of angiogenesis regulator release from platelet α-granules by conventional PKC inhibitors could affect angiogenesis, we performed capillary tube formation assays on Matrigel. Compared with controls, releasates from platelets inhibited by Ro 32-0432 (1 μM) or myr-FARKGALRQ (1 μM) significantly reduced the area covered by capillary-like structures (0.777 $\pm$ 0.161 mm^2 versus 0.490 $\pm$ 0.060 mm^2 and 1.563 $\pm$ 0.022 versus 1.397 $\pm$ 0.061, $P < 0.05$) (Fig. 5, A–C). Similarly, compared with controls, releasates from platelets inhibited by Ro 32-0432 (1 μM) or myr-FARKGALRQ (1 μM) significantly reduced the number of capillary-like structures branch points formed on the Matrigel (Fig. 5, D and E). Further, to confirm the antiangiogenesis effects of myr-FARKGALRQ on platelet releasates, we performed endothelial cell migration assays. Compared with controls, releasates from platelets inhibited by myr-FARKGALRQ (1 μM) were significantly weaker at stimulating HMVEC-L migration (226.8 $\pm$ 54.7 versus 126.5 $\pm$ 35.9 migrated cells per field of view, $P < 0.05$) (Supplemental Fig. 5).

Effect on Platelet Dense-Granule Release. Conventional isoform PKCs have been also reported to regulate dense-(δ)-granule release. Hence, to assess whether conventional PKC inhibitors at concentrations that uncoupled platelet aggregation from α-granule release also inhibit δ-granule secretion, we measured platelet ATP secretion in response to collagen. Compared with control, Ro 32-0432 (1 μM) significantly inhibited ATP release (100% versus 72.1% $\pm$ 10.2% versus 57.1% $\pm$ 11.7%, respectively) in response to collagen (Fig. 6A). However, myr-FARKGALRQ (1 μM) failed to significantly inhibit this release (100.0% $\pm$ 0.0% versus 79.5% $\pm$ 15.1%, $P >$ 0.05). (Fig. 6B). In general, both conventional PKC inhibitors, at concentrations that had minimal effects on aggregation, had an approximately 10–20% greater inhibitory effect on VEGF α-granule versus ATP δ-granule release.

To elucidate 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 PMA. PMA caused concentration-dependent release of both α- and δ-granules as measured by P-selectin flow cytometry and ATP secretion by chemiluminescence (Supplemental Fig. 6); however, α-granule release occurred at lower concentrations of PMA than δ-granule release (P-selectin surface exposure EC_{50} 1.03 nM versus ATP release EC_{50} 125.5 nM).

Discussion

The major novel finding of our study implicates PKCα in regulating angiogenesis-promoting properties of human platelets. Numerous studies have now shown potent angiogenesis stimulatory effects of platelets and their associated angiogenesis regulators, which are secreted upon aggregation (Jurasz et al., 2003; Brill et al., 2004; Battinelli et al., 2011; Radziwon-Balicka et al., 2012a). This platelet activity plays an important role in regulating angiogenesis under physiologic and pathologic conditions such as carcinogenesis (Verheul et al., 1997; Pietramaggiori et al., 2008; Klement et al., 2009). However, to date, platelet signaling pathways that control secretion of platelet-associated angiogenesis regulators from their granules have not been widely investigated. We

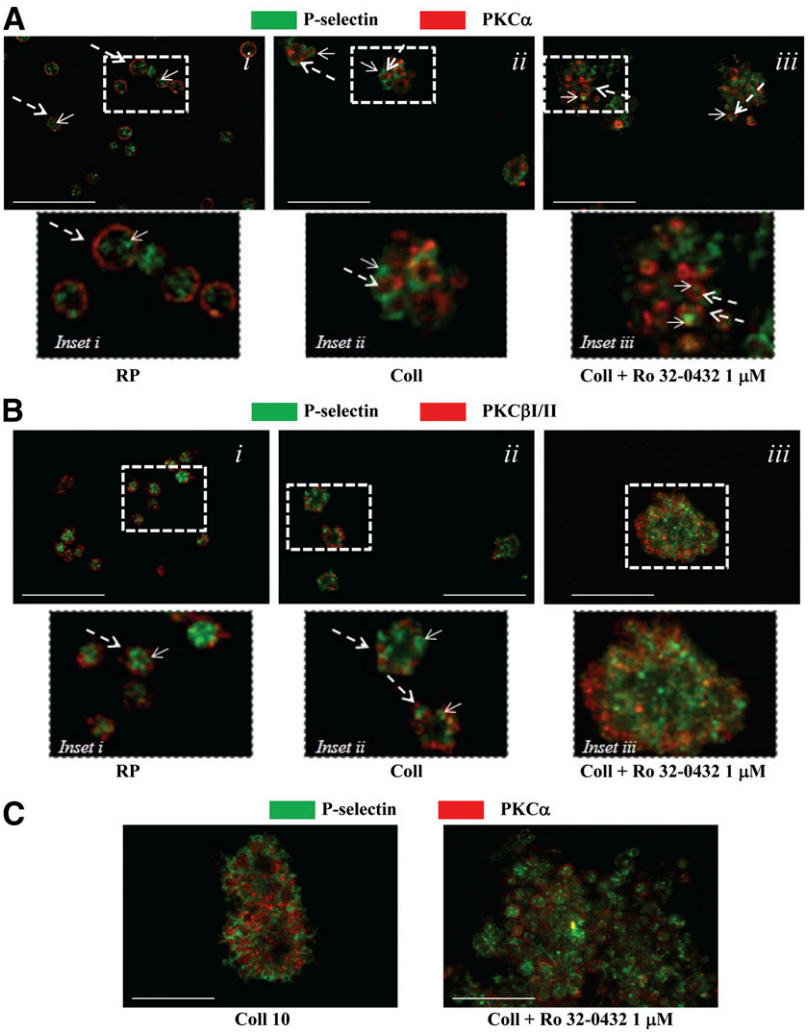

Fig. 3. Selective PKCα inhibition prevents α-granule release but not aggregation. (A*i*) Immunofluorescence confocal microscopy of PKCα and P-selectin (α-granule marker) in resting platelets. (A*ii*) Translocation of PKCα to centralized granules of small collagen (10 μg/ml)-induced platelet aggregates. Conversely, P-selectin translocates from α-granules to the platelet surface. (A*iii*) Ro 32-0432 (1 μM) prevents PKCα translocation to centralized granules and P-selectin release onto the platelet surface in small aggregates. (B*i–iii*) PKCβ immunostaining in resting, collagen-aggregated, and Ro 32-0432 (1 μM) inhibited platelets. (C) Immunofluorescence microscopy demonstrating the PKCα translocation inhibitory effects of Ro 32-0432 (1 μM) in large aggregates. Representative images are from three independent experiments. Large dashed arrows indicate either PKCα or β. Small arrows indicate P-selectin. Scale bars represent 16 μm. Coll, collagen; RP, resting platelets.

determined the effects of PKC signaling on angiogenesis regulator release from platelet α-granules and consequently on platelet-stimulated angiogenesis. Previous studies had shown PKCs, particularly the conventional isoforms α and β, to be important mediators signaling both platelet aggregation and α-granule release (Yoshioka et al., 2001; Tabuchi et al., 2003; Buensuceso et al., 2005; Konopatskaya et al., 2009). Although a certain signaling overlap between these two isoforms has been described, recent evidence suggests there may be a degree of nonredundancy in signaling with PKCα mediating platelet secretion while PKCβ participates in integrin αIIb/β3 activation (Gilio et al., 2010). If this subtle level of signaling control exists in human platelets, we hypothesized that it may be possible to uncouple platelet aggregation from α-granule release by selectively inhibiting PKCα, which would result in impaired secretion of platelet-associated angiogenesis regulators and platelet-stimulated angiogenesis.

Having established that P-selectin surface exposure was a good marker of α-granule release and a surrogate marker of released platelet-associated angiogenesis regulators in our experimental system, we screened several PKC inhibitors for their aggregation-α-granule uncoupling effects. We found that titration of the small molecule inhibitor Ro 32-0432 (Wilkinson et al., 1993) identified a concentration (1 μM) that inhibited α-granule release but not aggregation. Similar to Ro 32-0432, the membrane permeable inhibitory pseudosubstrate peptide mimetic myr-FARKGALRQ, which corresponds to a nine amino acid pseudosubstrate region of both PKCα and β (Kubo et al., 1987), also inhibited α-granule release but not aggregation at 1 μM. A previous study has shown that myr-FARKGALRQ inhibits myristoylated alanine rich C-kinase substrates phosphorylation in human fibroblasts with an 8 μM IC50, while in the absence of myristoylation the peptide is two orders of magnitude less effective at crossing the plasma membrane and inhibiting PKC (Eichholtz et al., 1993). Myristoylation plays a fundamental role targeting proteins to membranes. Hence, myr-FARKGALRQ at the low concentration of 1 μM likely preferentially targets and inhibits platelet membrane-associated PKCα over PKCβ, which we found to be primarily cytosolic. Paradoxically, myr-FARKGALRQ at concentrations greater than 10 μM potentiated collagen-induced aggregation (unpublished data). This aggregation potentiating effect is consistent with previous findings that demonstrated that high concentrations of pseudosubstrate peptide inhibitors containing the FARKGALRQ sequence inhibit an ecto-PKC on the platelet surface membrane that maintains fibrinogen receptor latency and hence whose inhibition enhances aggregation (Babinska et al., 1996, 2000). Thus, one of the

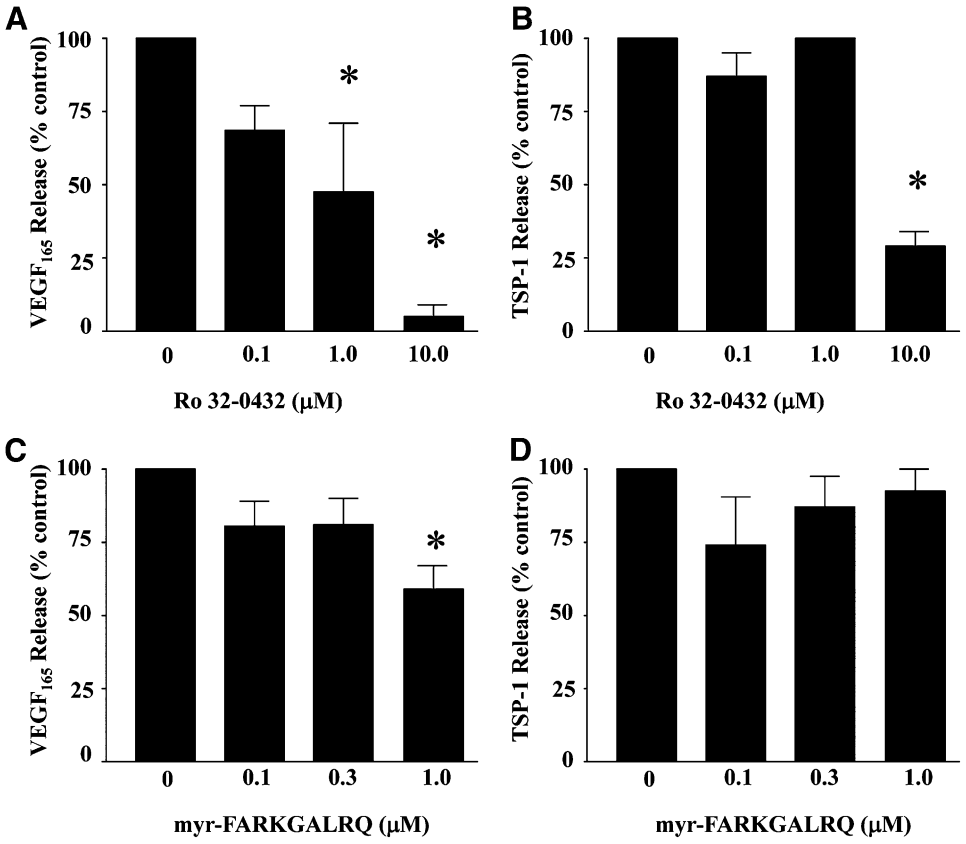

Fig. 4. Conventional PKC inhibitors suppress VEGF release from platelets. Effects are shown of conventional PKC inhibitors Ro 32-0432 (A and B) and myr-FARKGALRQ (C and D) on VEGF and TSP-1 release from collagen (10 μg/ml)-aggregated platelets. N = 5–9 experiments. *$P < 0.05$ versus control.

limitations of the pharmacologic tools that we have used in our studies is the potential for "off-target" activities, particularly at high concentrations. In addition, to myr-FARKGALRQ having off-target effects at high concentrations, Ro 32-0432 has been reported to also inhibit PKCδ (Li et al., 1999) and several G-protein-coupled receptor kinases (GRKs) (Aiyar et al., 2000). Although we cannot completely discount off-target effects, we observed aggregation-α-granule uncoupling at lower concentrations than those reported to inhibit these other kinases (1 versus 10 μM). Moreover, rottlerin, which includes PKCδ among its targets, failed to preferentially inhibit α-granule release over aggregation. If Ro 32-0432 were acting by inhibiting GRKs, platelet responses to agonists would be expected to be enhanced and not inhibited because GRKs are known to desensitize P2Y12 receptors (Hardy et al., 2005), which ultimately signal ADP-mediated α-granule release. Interestingly, we did not observe the aggregation-α-granule uncoupling effect with the nitric oxide donor GSNO (Jurasz et al., 2001a), a broad-spectrum platelet inhibitor, nor with the PKCβ inhibitor. Hence, the aggregation-α-granule uncoupling effect is likely limited to selective PKCα inhibitors within a narrow concentration range.

To determine which of the conventional PKC isoforms is antagonized when platelet aggregation is uncoupled from α-granule release, we performed immunofluorescence confocal microscopy to track "PKC targeting" (Shirai and Saito, 2002) within platelets. Competent PKC catalytic activity is necessary for a series of serine/threonine autophosphorylation events that are required for correct PKC subcellular localization/targeting (Newton, 2001; Shirai and Saito, 2002). Hence, we chose to track disruptions in PKCα and β targeting to determine which of the two conventional PKC isoforms is antagonized when α-granule release is uncoupled from aggregation. We found that normally upon aggregation PKCα translocated to the centralized α-granules of activated platelets while P-selectin translocated from α-granules to the platelet surface membrane. In the presence of an aggregation-α-granule uncoupling concentration of Ro 32-0432 (1 μM), PKCα failed to target centralized α-granules, and consequently P-selectin did not translocate from α-granules to the platelet membrane surface in many of the platelets. Ro 32-0432 exerted no discernible effect on PKCβ targeting. Taking into consideration the PKC targeting data and the fact that the PKCβ inhibitor 3-(1-[3-imidazol-1-ylpropyl]-1*H*-indol-3-yl)-4-anilino-1*H*-pyrrole-2,5-dione failed to uncouple α-granule release from platelet aggregation, we propose that the inhibition of PKCα but not β results in uncoupling of α-granule release from aggregation. The uncoupling of platelet secretory function from aggregate formation is not entirely without precedence. Indeed, an early study showed that the PKC agonist 12-*O*-tetradecanoyl phorbol-12-acetate can induce ATP secretion from platelet δ-granules even when platelet aggregation is suppressed by aspirin and a lack of external calcium (Rink et al., 1983). Our data demonstrate the opposite uncoupling (suppression of secretion and maintenance of aggregation) with PKC signaling once again regulating distinct platelet functions. Our results are in line with those of Flaumenhaft's group who have recently shown that pharmacologic inhibition of platelet actin polymerization abolishes α-granule secretion but not aggregation (Woronowicz et al., 2010).

Having established that selective PKCα inhibition uncouples α-granule release from aggregation, we investigated

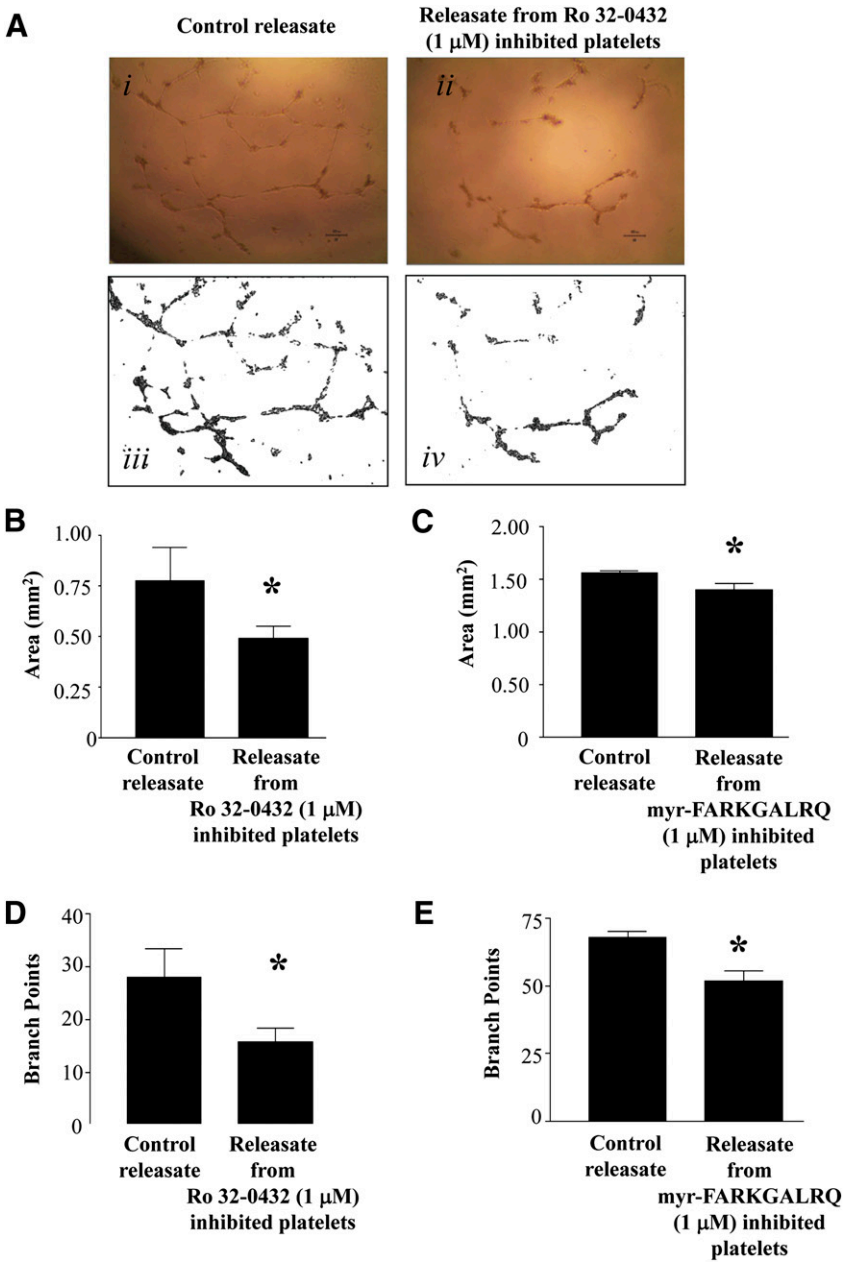

Fig. 5. Effects of conventional PKC-inhibited platelet releasates on the formation of capillary-like structures by human microvascular endothelial cells after 24 hours on Matrigel. (A) Representative microscopy (A*i* and *ii*) and binary (skeletonized) images (A*iii* and *iv*) of collagen (10 μg/ml)-aggregated control releasates and releasates from Ro 32-0432-(1 μM)-inhibited platelets. Skeletonized images were used to determine the total area covered by capillary-like structures. (B and C) Summary data of area covered by capillary-like structures in response to releasates from (B) Ro 32-0432 (1 μM) or (C) myr-FARKGALRQ (1 μM)-inhibited platelets. (D and E) Summary data of capillary-like structure branch points in response to releasates from (D) Ro 32-0432 (1 μM) or (E) myr-FARKGALRQ (1 μM)-inhibited platelets. $N = 4$. $^*P < 0.05$ versus control.

whether this phenomenon would result in impaired release of platelet-α-granule–associated angiogenesis regulators and platelet-stimulated angiogenesis. Because platelets contain both proangiogenesis and antiangiogenesis regulating factors that have been shown to be localized in distinct α-granules and differentially released (Ma et al., 2005; Italiano et al., 2008), we focused on VEGF and TSP-1, two prototypical proangiogenesis and antiangiogenesis regulating factors that are stored in α-granules. Ro 32-0432, a bisindolylmaleimide that competes with ATP for PKC binding (Wilkinson et al., 1993), preferentially inhibited VEGF over TSP-1 release. We found that myr-FARKGALRQ, an inhibitor that prevents PKC substrate binding, only inhibited VEGF release. One possible explanation for these findings is that PKCα has a differential affinity for phosphorylating substrates such as soluble NSF [N-ethylmaleimide sensitive factor] attachment protein receptors (Polgar et al., 2003; Ren et al., 2008) that mediate the exocytosis of α-granules containing proangiogenesis versus antiangiogenesis regulating factors. A second possibility is that kinases other than PKCα may be involved in regulating the release of α-granules or their compartments containing TSP-1, and that Ro 32-0432 at high concentrations (10 μM) inhibits other off-target kinases such as Syk, Janus kinase 2, or protein kinase D (Konopatskaya et al., 2011) that may be involved in TSP-1 release. A third possibility is that the release of α-granules or α-granule compartments (Kamykowski et al., 2011) that store antiangiogenesis regulating factors such as TSP-1 is coupled to platelet aggregation.

Interestingly, rottlerin, similar to myr-FARKGALRQ, inhibited VEGF but not TSP-1 release. However, unlike myr-FARKGALRQ, rottlerin was a more potent inhibitor of platelet aggregation than VEGF release. Surprisingly, our

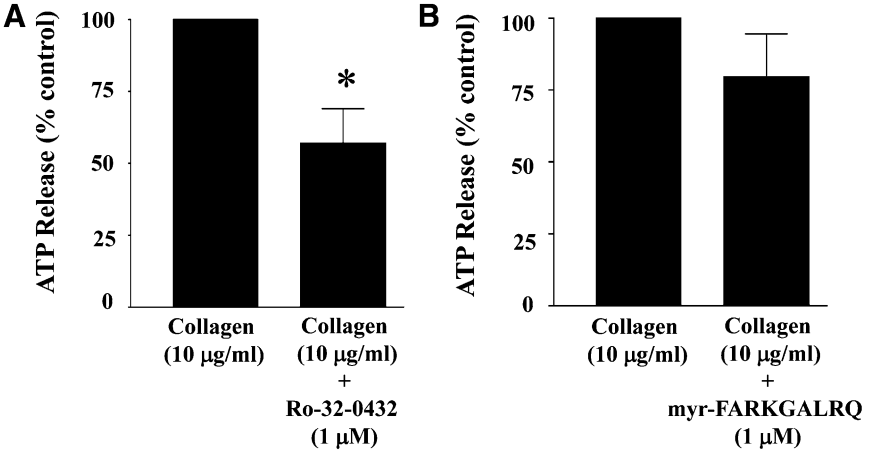

Fig. 6. Effects of conventional PKC inhibitors on platelet dense-granule release. Summary data demonstrate the effects of (A) Ro 32-0432 (1 μM) and (B) myr-FARKGALRQ (1 μM) on ATP release from platelet δ-granules. $N = 3–5$. $^*P < 0.05$ versus control.

findings with rottlerin contrast with those of other studies which have shown that in the presence of platelet cyclooxygenase inhibition rottlerin enhances collagen-induced platelet aggregation (Pula et al., 2006) and convulxin-induced δ-granule secretion (Murugappan et al., 2004). A possible explanation for this discrepancy is that our washed platelet preparations did not contain cyclooxygenase inhibitors.

Because PKC inhibitors can alter the balance between VEGF and TSP-1 release, we investigated their effects on platelet-stimulated angiogenesis in vitro. Releasates from Ro 32-0432– and myr-FARKGALRQ–inhibited platelets were weaker at promoting platelet-stimulated angiogenesis than controls. These results may be explained by the ability of Ro 32-0432 and myr-FARKGALRQ to lower the ratio of VEGF versus TSP-1 released from platelets, and thus perhaps inhibit the overall platelet-angiogenic balance (Hanahan and Folkman, 1996).

Finally, because PKCα signaling has been shown to also mediate δ-granule secretion (Konopatskaya et al., 2009), we investigated whether conventional PKC inhibitors at concentrations that uncouple angiogenesis regulator release from aggregation also impair δ-granule secretion. Ro 32-0432 suppressed δ-granule release as measured by secreted ATP; however, the effects on ATP secretion were generally less than on VEGF release from α-granules. Consistent with another study that showed myr-FARKGALRQ to a have minor inhibitory effect on δ-granule secreted serotonin (Wheeler-Jones et al., 1995), myr-FARKGALRQ did not have significant inhibitory effects on δ-granule release. To help explain why conventional PKC inhibitors in general had greater α-granule versus δ-granule suppressive effects, we demonstrated that platelet α-granule release is more susceptible to PKC activating stimuli than δ-granule release. Thus, it is likely that when α-granule release is uncoupled from aggregation enough ADP is secreted from δ-granules to maintain aggregation.

In summary, we have shown that selective PKCα inhibition uncouples the release of platelet α-granule-associated angiogenesis regulators from aggregation and this suppression of α-granule release impairs platelet-stimulated angiogenesis. The clinical significance of these findings still needs to be studied. However, in principle, platelet PKCα inhibition may be of benefit to reduce the effects of platelets on cancer angiogenesis. Indeed, such treatment could be used as an adjunct to platelet transfusions in cancer patients with chemotherapy-induced thrombocytopenia. Cancer patients have activated hemostatic systems, and platelet transfusions restore one of the largest circulating reservoirs of angiogenesis promoters. Hence, treatment of platelets with low concentrations of a PKCα inhibitor before transfusion could potentially limit the release of platelet-associated angiogenesis regulators but still maintain hemostasis.

Author Contributions

Participated in research design: Moncada de la Rosa, Ruvolo, Radomski, Jurasz.

Conducted experiments: Moncada de la Rosa, Radziwon-Balicka, El-Sikhry, Ruvolo, Jurasz.

Performed data analysis: Moncada de la Rosa, Radziwon-Balicka, Ruvolo, Jurasz.

Wrote or contributed to the writing of the manuscript: Moncada de la Rosa, Seubert, Ruvolo, Radomski, Jurasz.

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Address correspondence to: Paul Jurasz, Faculty of Pharmacy and Pharmaceutical, University of Alberta, 3142E Katz Group-Rexall Centre for Pharmacy & Health Research, 11361–87 Avenue, Edmonton, Alberta, Canada, T6G 2E1. E-mail: Jurasz@ualberta.ca