Targeting of Type I Protein Kinase A to lipid rafts is required for platelet inhibition by the cAMP-signalling pathway.

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Abstract

Background: cAMP signalling modulates platelet adhesion to von Willebrand factor (vWF) through protein kinase A (PKA)-mediated phosphorylation of glycoprotein (GP)Ibβ□□□A-kinase anchoring proteins (AKAPs) are proposed to control the localization and substrate specificity of individual PKA isoforms. However, the role of PKA isoforms in regulating the phosphorylation of GPIbβ and platelet response to vWF is unknown.

Objectives: We wished to determine the role of PKA isoforms in the phosphorylation of GPIbβ and platelet activation by vWF as a model for exploring the selective partitioning of cAMP signalling in platelets.

Results: The two isoforms of PKA in platelets, type I (PKA-I) and type II (PKA-II), were differentially localised, with a small pool of PKA-I found in lipid rafts. Using a combination of Far Western blotting, immunoprecipitation, proximity ligation assay and cAMP pull down we identified moesin as an AKAP that potentially localises PKA-I to rafts. Introduction of cell-permeable anchoring disruptor peptide, RI anchoring disruptor (RIAD-Arg_{11}), to block PKA-I/AKAP interactions, uncoupled PKA-RI from moesin, displaced PKA-RI from rafts and reduced kinase activity in rafts. Examination of GPIbβ demonstrated that it was phosphorylated in response to low concentrations of PGI_2 in a PKA-dependent manner and occurred primarily in lipid raft fractions. RIAD-Arg_{11} caused a significant reduction in raft-localised phosphoGPIbβ and diminished the ability of PGI_2 to regulate vWF-mediated aggregation and thrombus formation *in vitro*.

Conclusion: We propose that PKA-I-specific AKAPs in platelets including moesin, organize a selective localisation of PKA-I required for phosphorylation of GPIbβ and contributes to inhibition of platelet vWF interactions.

Introduction

In healthy blood vessels excessive platelet activation is controlled by endothelial-derived prostacyclin (PGI_2) through the formation of 3',5'cyclic adenosine monophosphate (cAMP). PGI_2 binds to its Gαs-coupled IP receptor resulting in the activation of adenylyl cyclase and consequently the production of cAMP(1). Elevations in cAMP are associated with inhibition of a number of platelet functions including Ca^{2+} mobilisation, dense granule secretion, activation of integrin αIIbβ3 and aggregation (2–4). The foremost effector of cAMP signalling in blood platelets is protein kinase A (PKA), of which there are two isoforms PKA type I (PKA-I) and PKA type II (PKA-II)(5–7). The holoenzyme PKA is composed of two regulatory (R) and two catalytic (cat) subunits. The phosphorylation of numerous proteins by PKA, including Gα13(8), inositol trisphosphate receptor (IP_3R)(9), phosphodiesterase 3A (PDE3A)(10), RhoA(11), vasodilator activated phosphoprotein (VASP)(12), glycoprotein GPIbβ(13), CalDAG-GEF1(14) and potentially many others(7), is thought to be a major route of cAMP-mediated regulation of platelet function. However, localisation and relative contributions of the individual isoforms of PKA to the regulation of platelet function remain unknown.

In many cell types cAMP/PKA signalling pathways are spatially and temporally regulated to allow multiple cAMP signalling networks to operate simultaneously and regulate distinct biological functions. The isoform-specific spatiotemporal regulation of PKA signaling is mediated by a group of structurally diverse, but functionally similar proteins termed A-kinase anchoring proteins (AKAPs)(15,16). PKA isoforms bind AKAPs through a direct interaction between a conserved amphipathic helix motif within all AKAPs and the docking/dimerization domain (D/D domain) of the R dimers of the PKA holoenzyme(16), which then focuses kinase activity on specific substrates. AKAPs are designated as type I or type II depending on whether they preferentially interact with PKA-I or PKA-II. In the absence of isoform specific pharmacological inhibitors, the uncoupling of PKA-I and -II from their AKAP scaffolds by cell permeable peptides have been used as an alternative strategy to delineate PKA isoform-specific effects and the role of AKAPs(17–20). In this study, we used these peptides to study PKA-mediated phosphorylation of GPIbβ as a model for examining the potential compartmentalisation of cAMP signalling in platelets. Our data indicate that a pool of PKA-I is localised to lipid raft fractions through the AKAP moesin, where the kinase can target GPIbβ□

Experimental procedures

Materials

Anti-PKA-RI and PKA-RII antibodies were from BD biosciences (Oxford, UK). Anti-phospho-PKA substrate, recognising RRXpS/T PKA consensus motif and anti-moesin antibodies were from Cell Signalling Technology (Hitchen, UK). Anti-phospho-GPIbβ was a kind gift from Professor Xiaoping Du (University of Chicago). Anti-LAT, anti-β-tubulin and IgG control antibodies were from Upstate (Watford, UK). The anti-integrin β3 and anti GPIbβ were from Santa Cruz Biotechnology (Heidelberg, Germany). PGI_2 and cAMP immunoassay kits were from Cambridge Bioscience (Cambridge, UK) and vWF (Heamate P) was from CSL Behring. stHt31 and PKA assay kit were from Promega. Rp-8-CPT-cAMPS and 8AHA-2'-O-Me-cAMP-agarose beads were from Biolog (Breman, Germany). RIAD-Arg_{11} (LEQYANQLADQIILEATE–R_{11}), scrambled (scr) RIAD-Arg_{11} (IEKELAQQYQNADAITLE-R_{11}) and recombinant FLAG-RII were synthesised as previous described(21). All other chemicals, including the Duolink™ proximity ligation assay kit, were from Sigma (Poole, UK).

Preparation of washed platelets.

Blood was taken from drug-free volunteers by clean venepuncture using acid citrate dextrose (ACD; 29.9mM sodium citrate, 113.8mM glucose, 72.6mM NaCl and 2.9mM citric acid, pH 6.4) as anticoagulant. Washed platelets were prepared as described previously(11).

Platelet aggregation and flow studies

For aggregation studies, washed platelets ($2.5x10^8$ platelets/ml) were treated with PGI_2 and then stimulated with vWF/ristocetin under constant stirring conditions (900rpm) for 4minutes using a Chronolog light aggregometer. For aggregation under flow, washed platelets ($4x10^8$ platelets/ml) were treated with PGI_2, labelled with $DIOC_6$ (1µM) at 37°C for 10minutes and reconstituted with autologous washed red blood cells (50% v/v). Platelet accrual on vWF in reconstituted blood was examined as previously described(22). Where necessary platelets were incubated with PKA-AKAP inhibitory peptides for 1hour prior to addition of PGI_2.

Lipid raft preparation and subcellular fractionation

Platelet lipid rafts were isolated based on the method of Lee(23). In brief, washed platelets (450µl; $1x10^9$ platelets/ml) were lysed with 2x Triton-X100 lysis buffer (20mM Tris, 100mM NaCl, 60mM sodium pyrophosphate, 20mM sodium glycerophosphate, 0.02% w/v sodium azide, 0.065% Triton X-100, protease and phosphatase inhibitor cocktails, pH 8.0). Lysates were vortexed and incubated on ice for 30minutes. Samples were mixed with equal volumes of 80% (w/v) sucrose to give 40% (w/v)

final concentration. This was transferred to the bottom of an ultracentrifuge tube. 5ml of 30% (w/v) sucrose was layered on top followed by another 5ml layer of 5% (w/v) sucrose. Tubes were ultracentrifuged at 200,000*g* for 18hours at 4°C. Sequential twelve 1-ml fractions were collected from the top of each sample(23).

For subcellular fractionation washed platelets ($5x10^8$ platelets/mL) were lysed by addition of equal amount of 2X fractionation buffer supplemented with protease and phosphatase inhibitors followed by 5 freeze-thaw cycles. Lysates were subjected to ultracentrifugation at 100,000*g* for 90minutes at 4°C. Supernatants (cytosolic fraction) were aspirated and pellets (plasma and intracellular membranes) were resuspended with IP lysis buffer(11). Protein concentrations were measured and equal amounts of protein were analysed. Fractionated platelet lysates were subjected to SDS-PAGE and immunoblotting. β3 was used to validate the fractionation. For quantification of raft proteins, LAT in fraction 5 was used as a reference for equal loading.

Immunoprecipitation, cAMP pull down and immunoblotting.

Washed platelets ($3-5x10^8$ platelets/ml) were incubated with apyrase (2U/ml), indomethacin (10μM) and EGTA (1mM) before treatment with PGI_2. Platelets were lysed with Laemmli buffer and proteins were separated by SDS-PAGE before transfer to PVDF membranes. In some cases, platelets were incubated with RIAD-Arg_{11}/scrRIAD-Arg_{11} or PKA inhibitors before addition of PGI_2. For immunoprecipitation, washed platelets ($8×10^8$ platelets/ml) were lysed with ice-cold lysis buffer and proteins were immunoprecipitated using standard protocols(11). Proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for 60minutes with 10% BSA in Tris-buffered-saline-Tween (0.1%) (TBS-T), then incubated with the indicated antibody. Immunoblots were processed as described previously(11).For cAMP pull down experiments, platelet lysates were incubated with agarose-beads linked to 8AHA-2'-O-Me-cAMP, which show greater specificity for PKA-RI(24). Beads were incubated with lysates overnight at 4°C with mixing. Beads were then washed and proteins eluted by boiling in Laemmli buffer for 5minutes and analysed by SDS-PAGE followed by immunoblotting. In some cases, Coomassie-stained gel bands were cut out, trypsin-digested and then analysed by MALDI-TOF mass spectrometry.

PKA activity assay

PKA activity was measured by phosphorylation (increase negative charge) of the synthetic substrate kemptide (Supplementary method 1).

PKA-RI Overlay Assay

R overlays were conducted as previously described(25,26), using bacterially expressed and purified FLAG-PKA-RI. Briefly, platelet lysates were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% skimmed dry milk and 0.1% BSA in TBS-T and then incubated with FLAG-PKA-RI, in the presence or absence of stHt31 (20µM), overnight at room temperature with agitation. Membranes were washed and immunoblotted with an anti-FLAG antibody. Cellular proteins not binding to recombinant RI in the presence of 20µM stHt31 were considered putative AKAPs.

cAMP measurement

Washed platelets ($2x10^8$platelets/ml) were treated with PGI_2 (50nM) for 1minute, in the presence and absence of inhibitors, before termination with lysis buffer. cAMP levels were assayed with a commercial enzyme immunoassay system and expressed as fmol cAMP/$1x10^7$ platelets.

Immunofluorescence and Duolink™ proximity ligation assay

The cellular distribution of PKA isoforms and their interactions with other proteins were visualised by standard immunofluorescence or in combination with a Duolink™ proximity ligation assay (PLA)(27) as described in supplementary method 2.

Statistical analysis

Results are expressed as means ± SEM and were analysed using the Student's t-test or ANOVA. The results were considered significant when p values were < 0.05.

RESULTS

A population of PKA-I is localized to platelet lipid rafts

To begin to understand how cAMP signaling is localised in blood platelets we examined PKA phosphorylation events in distinct cellular compartments using an antibody that recognizes phosphorylated PKA substrates. Under basal conditions distinct phosphoproteins were observed in both particulate and soluble fractions (Figure 1A). Treatment with PGI_2 (100nM) increased phosphorylated proteins in the particulate fraction with apparent molecular weights of 16, 24, 27, 40, 48, 55, 65, 70, 100 and 120kDa. While in the soluble fractions protein with apparent molecular weights of 18, 27, 35, 50, 60, 70, and 120kDa were observed, suggesting that PKA may target distinct substrates in different compartments in platelets. Immunoblotting for PKA-I and PKA-II regulatory subunits (PKA-RI and PKA-RII) in platelet fractions showed that PKA isoforms were present in both membrane and cytosolic fractions, but that PKA-I was more prominent in membrane fractions in comparison to PKA-II (supplementary figure 1). Consistent with the subcellular fractionation data, confocal microscopy showed that under resting conditions PKA-RI was found primarily at the periphery of the cell, whereas PKA-RII staining was localised in clusters within the cytosol (Figure 1Bi and Bii).

Recently we found a pool of AC5/6 localized in lipid rafts(28) suggesting that these cholesterol and sphingolipid enriched microdomains could contribute to cAMP signaling in platelets(28). To examine if PKA was localised to these domains, platelet lysates were separated into lipid raft and non-lipid raft fractions by sucrose gradient ultracentrifugation and analysed by SDS-PAGE and immunoblotting. The detergent-resistant fraction was enriched in LAT, an established raft marker, whereas the non-raft protein integrin β3 was excluded (Figure 1C)(23,29). Under basal conditions PKA-RI was found primarily in non-raft fractions, with a modest, but significant pool of PKA-RI found in the lipid raft fractions (Figure 1C and supplementary fig 2). In contrast, PKA-RII was found exclusively in non-raft fractions. Elevated cAMP concentrations triggered by PGI_2 (100nM) caused small but significant increase in raft-localised PKA-RI, but had no effect on PKA-RII and LAT(Figure 1C). These data suggest that there is distinct localisation of PKA isoforms in platelets with PKA-RI the only isoform identified in lipid rafts.

Moesin is an AKAP that binds PKA-I in platelets

The localization of PKA isoforms is organized by AKAPs(15). To determine whether AKAPs were important for PKA-I localisation we adopted a four-staged strategy. Firstly, PKA-I binding proteins in platelets were screened using RI-overlay assay (Far Western blotting) in the presence or absence of StHt-31, a peptide inhibitor of PKA-AKAP interactions, to identify putative AKAPs(25). Incubation of recombinant FLAG-PKA-RI with immobilised platelet lysates revealed five potential AKAPs with

apparent molecular masses of approximately 37, 42, 80, 100 and 140kDa (Figure 2A). We confirmed these findings by cAMP pull down (supplementary figure 3B). We were particularly interested in the 80kDa band since it was the most consistent and because the Ezrin-Radixin-Moesin (ERM) family member Ezrin (80kDa) had previously been identified as an AKAP that binds PKA-RI in T-cell lipid rafts(30). Further investigation by immunoblotting showed that the band at approximately 80kDa was the membrane-cytoskeleton binding protein moesin (Figure 2Aii), which was confirmed by mass spectrometry (data not shown). Moesin has been identified as an AKAP previously(31,32) and therefore the second stage of our strategy sought to confirm that moesin binds PKA-RI in platelets. Using a cAMP pull-down to precipitate PKA-RI isoforms and associated binding proteins(24), we found that moesin was precipitated and therefore was potentially associated with PKA-RI (Figure 2B). While the cAMP coated beads we used have a higher specifically for RI than RII they do not fully discriminate between the two isoforms (Figure 2B) and so in a third approach we found that immunoprecipitation of PKA-RI from platelets led to the co-precipitation of moesin (Figure 2C). This association was confirmed by reverse immunoprecipitation using a moesin antibody, demonstrating the presence of PKA-RI and the associated PKAcat (Figure 2D and supplementary figure 4). As expected only a small fraction of the moesin was associated with PKA-RI. We also used a cell permeable peptide inhibitor that uncouples PKA-AKAP interactions. Several peptides have been developed to examine PKA-AKAP interactions including Ht31, RI anchoring disruptor (RIAD-Arg_{11}) and SUPER-AKAP*IS*(21,33). However, we used RIAD-Arg_{11} because of its 1000-fold greater specificity for disruption of PKA-I- compared to PKA-II-AKAP complexes(21,33). Incubation of platelets with FITC-RIAD-Arg_{11}, but not FITIC-RIAD (without the polyarginine tail) exhibited strong fluorescence (supplementary figure 5) consistent with cell penetration. Pretreatment of platelets for 60minutes with RIAD-Arg_{11}, but not scrRIAD-Arg_{11}, reduced the amounts of moesin co-immunoprecipitated with PKA-RI suggesting that an interaction between the D/D domain of PKA-I and an amphipathic helix of moesin mediates their association (Figure 2C). In the fourth stage, we examined moesin-PKA-I association in intact cells using a Duolink™ proximity ligation assay to visualise localisation of interacting proteins. Using immunostaining-tested antibodies for PKA-RI and moesin (Data not shown) and wheat germ agglutinin as a membrane marker we detected PLA signal around the periphery of the cell suggesting that the two proteins are associated near platelet plasma membrane (Figure 2E). Together these data suggest that moesin is localised with PKA-RI and acts as PKA-RI binding protein in platelets.

PKA-I localises in lipid rafts through an AKAP-dependent mechanism

Next we wanted to examine whether the localization of PKA-RI to lipid rafts could be AKAP-mediated. To achieve this we disrupted PKA-I/AKAP interactions with RIAD-Arg_{11} and then examined lipid raft fractions. RIAD-Arg_{11} (10μM) did not affect the distribution profiles of LAT into lipid rafts, indicating that the raft fractions remained intact (Figure 3Ai, top panels). However, RIAD-Arg_{11} displaced a significant amount of the regulatory subunit of PKA-I from the detergent-resistant fractions ($p<0.05$; Figure 3A). In contrast, scrRIAD-Arg_{11} (10μM) had no effect on the localization of PKA-RI under the same conditions. Similar results were obtained with StHt31 (data not shown). The data also show that RIAD-Arg_{11} (10μM) did not affect the localization of moesin (Figure 3A – bottom panels), which is present in small amounts in lipid raft fractions. Consistent with raft localization of the complex the cholesterol-chelating agent MβCD, which disrupts lipid rafts, depleted PKA-RI and moesin from these fractions. cAMP pull down demonstrated that moesin was precipitated from rafts fractions as part of a PKA-RI complex. No moesin or PKA-RI was detected when lipid rafts were disrupted with MβCD (Figure 3B). Finally, we reasoned that if moesin was an AKAP binding to PKA then it should possess an associated PKA activity(34). Moesin was immunoprecipitated from lipid raft fractions and the ability to phosphorylate kemptide, a synthetic PKA substrate, examined. Immunoprecipitated moesin possessed a 3-fold higher PKA activity than with IgG (control) immunoprecipitates (Figure 3C). These data suggest that moesin in lipid rafts forms a complex with a pool of PKA-I that can facilitate membrane compartmentalization of the kinase.

Lipid raft localised GPIbβ is phosphorylated on serine^{166} by PKA-I

We next examined the importance of raft localised PKA-I to platelet activatory signaling and function. PGI_2 (100nM) induced the phosphorylation of proteins with apparent molecular weights of 27, 100 and 165kDa and increased the phosphorylation of the basally phosphorylated 75kDa protein (Figure 4A) in lipid rafts, confirming kinase activity in these fractions. The phosphorylation of these proteins was reduced significantly when platelets were treated with RIAD-Arg_{11} (10μM) and abolished by the PKA inhibitors KT5720/Rp-8-CPT-cAMPS (10μM/500μM).

GPIbβ is a PKA substrate, whose phosphorylation on serine166 leads to reduced vWF-mediated aggregation and adhesion to immobilized vWF and is found in lipid rafts(13,35,36). Using a specific antibody for GPIbβ we detected a band of approximately 27kDa, which corresponded to one of the bands detected using the phosphoPKA substrate antibody in lipid rafts. To investigate if GPIbβ could represent a PKA raft-localised substrate we used an antibody specific to phospho-GPIbβ-ser^{166}. Consistent with previous reports we observed basal phosphorylation of GPIbβ in whole cell lysate(13,37). Phosphorylation was increased by PGI_2 in a dose dependent manner (10-100nM) and restricted to basal levels by the PKA inhibitors KT5720/Rp-8-CPT-cAMPS (10μM/500μM) (Figure 4B).

While PKA-mediated phosphorylation of GPIbβ is associated with reduced platelet adhesion to vWF, the role of specific PKA isoforms in this process is unknown. In the absence of isoform-specific inhibitors or PKA-I deficient mice we used RIAD-Arg_{11} to simultaneously evaluate the role of AKAP coupling to PKA isoforms and partially isolate PKA-I signalling events(19,20,38). Under basal conditions we found no evidence of GPIbβ phosphorylation in lipid raft fractions (Figure 4C). However, treatment of platelets with PGI_2 (100nM) led to significantly increased phosphoGPIbβ in lipid rafts ($p<0.001$; Figure 4C), with some minor effects in the non-raft fraction. The presence of RIAD-Arg_{11} (10μM), but not scrRIAD-Arg_{11}, reduced the phosphorylation of raft-localized GPIbβ (Figure 4C). In contrast, no changes in phosphorylation of GPIbβ in non-raft fractions was detected. As expected a combination of PKA inhibitors, KT5720/Rp-8-CPT-cAMPS (10μM/500μM), abolished PGI_2 stimulated phosphorylation in lipid rafts. Finally, we confirmed that the inhibition of PGI_2-stimulated phosphorylation of GPIbβ by RIAD-Arg_{11} was at the level of PKA and not cAMP availability. Basal cAMP concentrations (87±37fmol/1x10^7 platelets) were increased to 150±52fmol/1x10^7platelets ($p<0.05$) by PGI_2 (50nM). Pretreatment of platelets with RIAD-Arg_{11} (10μM) or scrRIAD-Arg_{11} prior to stimulation with PGI_2, failed to affect cAMP synthesis (Figure 4D). These data could suggest that the phosphorylation of a population of GPIbβ in platelet lipid rafts is mediated by PKA-I and potentially requires its localization by an AKAP.

Disruption of PKA-I anchoring diminishes the inhibitory effect of PGI_2 on vWF-induced platelet aggregation and accrual under flow.

Next we investigated how PKA-I uncoupling influenced platelet function. PGI_2 (50nM) reduced vWF (20μg/mL)/ristocetin(0.75mg/ml)-mediated aggregation from 78.9±4.7 to 34±5%, but remained elevated at 61.8±5.8% ($p<0.01$, compared with PGI_2 alone; Figure 5A) in the presence of RIAD-Arg_{11} (2 □M). RIAD-Arg_{11} had no effect. In control experiments, RIAD lacking the poly-Arg tail (not cell permeable) or the poly-Arg tail alone failed to affect the inhibition of aggregation PGI_2 (data not shown). To further confirm the role of AKAPs we used the alternative cell permeating PKA-AKAP disruptor, stHt31 (Figure 5B), which disrupts anchoring of both type I and type II AKAPs(18). PGI_2 (50nM) reduced vWF/ristocetin-induced aggregation from 78.7±5.5% to 47.3±2% ($p<0.05$) (Figure 5B), although this elevated remained at 68.3±9% ($p<0.05$, compared to PGI_2 alone; Figure 5B) with stHt31 (2μM). In control experiments, the proline substituted stHt31P peptide (2μM) did not influence PGI_2-mediated inhibition (Figure 5B). In a series of control experiments we found that RIAD-Arg_{11} did not induce aggregation, potentiate aggregation responses induced by low dose vWF or inhibit responses induced by high-dose vWF (Figure 5C). Similar data were found with collagen or thrombin (data not

shown). These data indicate that the reduced sensitivity to PGI_2 in Figure 5A and 5B was likely due to inhibition of PKA stimulated signaling, rather than direct platelet activation by RIAD-Arg_{11} alone.

To determine if PKA-I-AKAP interactions influenced platelet function under physiological conditions, platelet aggregation was examined under flow. Under arterial shear ($1000s^{-1}$), immobilized vWF (100µg/ml) supported adhesion of numerous small aggregates that covered 29.5±2% of the vWF-coated surface (Figure 6; supplementary video 1), which was reduced to 14±1.8% ($p<0.05$) by the presence of PGI_2 (100nM) (Figure 6; supplementary video 2). Treatment of platelets with RIAD-Arg_{11} (10µM) alone did not influence platelet accrual under flow (Figure 6; supplementary video 3), but reversed the inhibitory effects of PGI_2, with the level of surface coverage increasing to 21±3% ($p<0.05$ compared to PGI_2 alone) (Figure 6; supplementary video 4). In contrast, scrRIAD-Arg_{11} had no effect. Taken together these data suggest that uncoupling of PKA-I from potential platelet AKAPs reduces the ability of PGI_2 to inhibit vWF-mediated platelet aggregation and accrual under flow conditions.

Discussion

A significant body of evidence now indicates that cAMP-mediated regulation of cellular functions is facilitated through isoform-specific localisation of PKA(15). However, while cAMP signaling is an established mechanism for the inhibition of platelets, the PKA isoform-specific events have not been investigated. Elegant studies by *El Daher* and colleagues demonstrated PKA can target substrates distributed in different cellular compartments of platelets(39) while more recently the *Scholten* laboratory has provided evidence that cAMP signalling scaffolds and AKAPs are present in platelets(40,41). Taken together these data suggest a level of cAMP signal compartmentalisation exists in platelets. In this context, we present evidence of compartmentalisation of cAMP signalling that contributes to regulation of vWF-mediated platelet aggregation. We demonstrate that (i) a pool of PKA-I, but not PKA-II, is localised to membrane lipid rafts; (ii) moesin acts as a platelet AKAP that targets PKA-I to lipid rafts; (iii) raft-activated PKA-I phosphorylates GPIbβ and plays a key role in the inhibition of vWF-stimulated platelet functions (Figure7).

The role of individual PKA isoforms localised in distinct cellular compartments in cAMP signalling and regulation of platelet function has remained largely unexplored because of potential off targets of PKA inhibitors, the lack of isoform specific inhibitors or availability of PKA isoform null mutant mice. Therefore, the use of cell-permeable peptides that uncouple specific PKA isoforms from their AKAPs and partially isolate PKA isoform signalling events offered a strategy to begin to address this issue. Recently, we found that a pool of AC5/6 was located in lipid rafts suggesting that these microdomains played a role in cAMP signalling in platelets(28). Immunoblotting of lipid raft fractions demonstrated that a small pool of PKA-I, but not PKA-II, was present in rafts and was increased in response to elevated cAMP. Raft localisation of PKA-I was blocked by treatment of platelets with a cell permeable peptide designed to uncouple PKA-RI from AKAPs. Using a combination of far-western blotting, cAMP binding protein enrichment and immunoblotting we identified moesin as a PKA-RI binding protein, confirming the proteomic findings of a previous report (41). To examine if this association occurred in rafts we used a cAMP enrichment approach from raft fractions to isolate cAMP binding protein complexes(42,43). Immunoblotting of proteins precipitated from raft fractions by 8AHA-2'-O-Me-cAMP-agarose beads(24) demonstrated the presence of moesin. The confirmation that moesin was acting as an AKAP was provided by two further observations. Firstly, immunoprecipitated moesin was complexed with both PKA-RI and PKAcat and possessed PKA catalytic activity, and secondly, the association of moesin and PKA-RI was blocked by treatment of platelets with $RIADArg_{11}$, indicating that the interaction proceeded through the dimerization and docking domain of PKAR-RI. Together these data suggest that, consistent with other cell types, that moesin can act as an AKAP for PKA-RI in platelets(31,32).

Since only PKA-I was present in lipid rafts under the conditions investigated, we reasoned that proteins phosphorylated in response to PGI_2 in rafts were likely PKA-I targets. Using low concentrations of PGI_2 to replicate physiologically relevant signalling and an anti-phosphoPKA substrate antibody we found at least four substrates with apparent molecular weights of 27, 75, 100 and 165kDa in lipid rafts. A simple analysis of known PKA substrates in platelets suggested the 27kDa could represent GPIbβ, which is also found in rafts(35,36). Immunoblotting revealed GPIbβ was phosphorylated in lipid rafts in response to PGI_2 treatment in a PKA-dependent manner. Moreover, the uncoupling of PKA-I from raft fractions by RIAD-Arg_{11} led to diminished phosphorylation of raft GPIbβ, suggesting that phosphorylation of raft-localised GPIbβ is mediated by PKA type I and is facilitated by localisation of the kinase by an AKAP. The potential physiological significance of the AKAP-dependent PKA-I phosphorylation of the raft-residing GPIbβ, was demonstrated using vWF-mediated platelet aggregation and adhesion under flow. The uncoupling of PKA-I from its potential AKAPs partially reversed the inhibitory effect of PGI_2 on aggregation and platelets arrest under arterial flow. This approach would involve uncoupling multiple PKA-AKAP complexes in different cellular compartments, although the reduced of lipid raft phosphorylation of GPIbβ suggest that raft localisation of PKA-1 could be an important factor. Interestingly, the peptide inhibitor did not prevent fully the effects PGI_2 under any of the conditions tested. This is not unexpected since the inhibition of platelet activation by PGI_2 requires targeting of multiple aspects of platelet function, which likely involves both PKA type I and II. Since PKA-II would be insensitive to the uncoupling effects of RIAD-Arg_{11} it would still be able to contribute to cAMP mediated platelet inhibition.

Our data show the first evidence for the functional compartmentalization of PKA signalling in platelets. We propose that a pool of PKA-I is localised in platelet lipid rafts, potentially through interactions with the AKAP moesin and that this AKAP-anchored PKA-I facilitates the phosphorylation of lipid raft-residing GPIbβ. The mechanisms facilitating the raft localisation of moesin and PKA-I are still unclear, but may require other adaptor proteins as is the case for ezrin in T-cells (30). While it is likely that peptide disruptors such as RIAD-Arg_{11} uncouple numerous PKA-I-AKAP complexes in platelets, we believe our data demonstrate the principle of how compartmentalisation of cAMP signalling may be important to regulation of platelet function. However, it will be important in the future to examine the presence of both further PKA-I/AKAP and PKA-II/AKAP complexes and link these to the regulation of specific platelet functions.

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Conflict of interest.

None

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Figure Legends.

Figure 1: PKA isoforms have distinct subcellular localisation in platelets.

(A) Washed platelets ($5x10^8$ platelets/ml) in the presence or absence of PGI_2 (100nM, 1minute) were lysed and separated into soluble (S) and particulate (P) fractions. Fractionated lysates were separated by SDS-PAGE and blotted for phosphoPKA substrates, PKA-RI, RII and integrin β3. Arrows indicate bands that are increased in response to PGI_2. Immunoblots are representative of three independent experiments. **(B)** Washed platelets were fixed in-solution and spun down onto poly-L-lysine-coated glass coverslips. Platelets were then permeablised and stained with (i)anti PKA-RI, (ii)PKA-RII followed by Alexa fluor488-conjugated secondary antibody or (iii)WGA-Alexa fluor488. Images are representative of magnified platelets from three independent experiments. Scale bar: 2μM. **(C)** Washed platelets ($1x10^9$ platelets/ml) in the presence or absence of PGI_2 (100nM, 1minute) were lysed with 0.065% triton X-100 lysis buffer and separated into lipid raft and non-raft fractions by sucrose gradient ultracentrifugation. Fractions were separated by SDS-PAGE followed by immunoblotting with PKA RIα/β, RIIβ subunits, SLP76 and integrin β3. (i) Blots are representative of five independent experiments, (ii) fold change in PKA-RI in lipid raft and non-raft compartments (fraction 5 and 11, respectively). *$p<0.05$ compared to basal, NS: not significant.

Figure 2: Moesin is a PKA-RI binding protein in platelets.

(A)(i) Washed platelets ($1x10^9$ platelets/ml) were lysed and separated by SDS-PAGE. Resolved proteins were then transferred into a PVDF membrane and subjected to RI-overlay assay using recombinant FLAG-PKA-RI in the presence or absence of the anchoring disruptor StHt31 (20μM). Membranes were then immunoblotted with an antibody against the FLAG-tag. Arrow indicate potential PKA-RI-binding proteins in platelets. (ii) Untreated platelet lysate (WCL) proteins were resolved by SDS-PAGE and immunoblotted with anti-moesin antibody. Blots are representative of three independent experiments. **(B)** Washed platelets ($1x10^9$ platelets/ml) were lysed and incubated with 8AHA-2'-O-Me-cAMP agarose beads (cAMP-Ag) or the negative control (EtOH-Ag; no cAMP) beads (20μl beads/1mg of protein) overnight at 4°C. Associated proteins were analysed by SDS-PAGE and immunoblotted for moesin, PKA-RI, PKA-RII and PKAcat. Blots are representative of four independent experiments. **(C)** Washed platelets ($5x10^8$ platelets/ml) were lysed and subjected to immunoprecipitation using an antibody against PKA-RI subunit. In some cases washed platelets were treated with PGI_2 (100nM for 1min), RIAD-Arg_{11} (10μM), scrRIAD-Arg_{11} (10μM) or vehicle for 60minutes, before immunoprecipitation of the PKA-RI subunit. Precipitates were analysed by SDS-PAGE and immunoblotting using a moesin and PKA-RI antibodies. Blots are representative of four independent experiments. **(D)** As in (C) except immunoprecipitation was carried out with a moesin antibody and

blotted for moesin, PKA-RI and PKAcat. Blots are representative of four independent experiments. **(E)** (i-v, i'-v') platelets were subjected to PLA to determine physical proximity of molecules as described in methods. Platelets were co-stained with moesin/PKA-RI antibodies (i') or matched IgG controls (iii'). PKA-RI alone (ii') and moesin alone (iv') have been included as negative controls for the PLA, whereas PKA-RI/PKAcat (v') was included as a positive control. Platelet membranes were visualised using wheat germ agglutinin conjugated to Alexa Fluor-488 (i-v) using fluorescence microscopy. Scale bar: 2μM.

Figure 3: PKA-I and moesin are localised to lipid rafts and form a complex

(A) Washed platelets ($1x10^9$ platelets/ml) were treated with RIAD-Arg_{11}(10μM), scrRIAD-Arg_{11} (10μM) for 60minutes or MβCD (5mM) for 30minutes. Platelets were lysed with 0.065% triton X-100 lysis buffer and separated into lipid raft and non-raft fractions by sucrose gradient ultracentrifugation. Fractions were analysed by SDS-PAGE followed by immunoblotting with PKA RIα/β, RIIβ subunits, LAT and moesin. (i) Blots are representative of four independent experiments. (ii) Densitometric analysis of the amount of the indicated proteins in lipid rafts (n=4). Data are represented as mean fold change over basal ± SEM, *p<0.05, NS: not significant. **(B)** Washed platelets ($1x10^9$ platelets/ml) were either treated with MβCD (5mM) for 30minutes or left untreated, lysed with 0.065% triton X-100 lysis buffer and separated into lipid raft and non-raft fractions by sucrose gradient ultracentrifugation. Fractions 5 and 11 were then incubated with 8AHA-2'-O-Me-cAMP agarose beads (cAMP-Ag) or the negative control (EtOH-Ag; no cAMP) beads (20μl beads/1mg of protein) overnight at 4°C. Associated proteins were analysed by SDS-PAGE and immunoblotted for moesin and PKA-RI. Blots are representative of four independent experiments. **(C)** Immunoprecipitates using anti-moesin or non-specific antibody (IgG) from raft fraction 5 were examined for the ability to phosphorylate the synthetic PKA substrate kemptide to measure associated PKA activity. The assay uses a positive (+ve) control of recombinant PKAcat to examine enzyme activity and a negative (-ve) control where recombinant PKAcat was omitted. (i) A representative agarose gel image. (ii) Quantification of PKA activity where data is expressed as % PKA activity compared with IgG and representative of three independent experiments (*p<0.05 moesin compared to IgG).

Figure 4: PKA-I phosphorylates GPIbβ in platelet lipid rafts in an AKAP-dependent manner.

(A)(i) Washed platelets ($1x10^9$/ml), in the presence and absence of PGI_2 (100nM, 1minute), were lysed with 0.065% triton X-100 lysis buffer and separated into lipid raft and non-raft fractions by sucrose gradient ultracentrifugation. In some cases platelets were incubated with RIAD-Arg_{11} (10μM, 60minutes) or KT5720/Rp-8-CPT-cAMPS (10/500μM, 15minutes). Lipid rafts were separated by SDS-PAGE followed by immunoblotting with phosphoPKA substrate antibody. (ii) whole cell lysates (WCL)

were immunoblotting for GPIbβ□Blots are representative of four independent experiments. **(B)** Washed platelets ($3x10^8$/ml) were treated with PGI_2 (0-100nM, 1minute), or KT5720/Rp-8-CPT-cAMPS (10/500µM, 15minutes) followed by PGI_2. Platelets were lysed then separated by SDS-PAGE and blotted with phosphoGPIbβ-ser166, phosphoVASP-ser157 and β-tubulin. (i) Blots are representative of three independent experiments. (ii) Densitometric analysis of phosphoGPIbβ of three independent experiments (n=3) and expressed as mean fold change over basal ± SEM. *p<0.05 for PGI_2 compared to basal or PGI_2 in the presence or absence of KT5720/Rp-8-CPT-cAMPS. **(C)** Washed platelets ($1x10^9$/ml) were left untreated, treated with PGI_2 (100nM, 1minute) alone, or in the presence of RIAD-Arg_{11} (10µM, 60minutes), scrRIAD-Arg_{11} (10µM, 60minutes) or KT5720/Rp-8-CPT-cAMPS (10/500µM, 15minutes). Platelets were lysed with 0.065% triton X-100 then separated into lipid raft and non-raft fractions by sucrose gradient ultracentrifugation. Fraction proteins were analysed by SDS-PAGE followed by immunoblotting with PKA-RI or phosphoGPIbβ-ser166 antibodies followed by the raft and non-raft markers LAT and integrin β3, respectively. (i) Representative blots of three independent experiments. (ii) Densitometric analysis of phosphoGPIbβ-ser166 present in lipid rafts and non-raft fractions (fraction 5 and 11, respectively) (n=3). Data are expressed as mean fold change ± SEM, *p<0.05. **(D)** Platelets ($2x10^8$/ml) were pre-incubated with vehicle, RIAD-Arg_{11} (10µM) or scrRIAD-Arg_{11} (10µM) for 60minutes followed by treatment with PGI_2 (50nM) (grey bars) or vehicle (black bars) for 1minute. cAMP levels were measured as per manufacturer's instructions and expressed as fmol cAMP/$1x10^7$ platelets. Data are presented as means ± SEM of 3 individual experiments. *p<0.05 PGI_2 treatment compared to basal cAMP.

Figure 5: PKA-AKAP uncoupling peptides modulate inhibition of platelet aggregation by PGI_2.

(A) Washed platelets ($2.5x10^8$/ml) were pre-incubated with RIAD-Arg_{11} (1-10µM), scrRIAD-Arg_{11} (10µM) or vehicle for 60minutes followed by addition of PGI_2 (50nM) for 1minute and then stimulated with vWF/Ristocetin (20µg/ml, 0.75mg/ml) for 4 minutes under stirring conditions (i) Representative aggregation traces, (ii) Collated data of four independent experiments expressed as mean ± SEM . # p<0.01 compared to absence of PGI_2; *p<0.05 compared to PGI_2 alone. **(B)** As in (A) except platelets were pre-treated with StHt31(2µM) or StHt31P(2µM, negative control) or vehicle for 60minutes, (i) Representative aggregation traces, (ii) Collated data of four independent experiments expressed as mean ± SEM . # p<0.01 compared to absence of PGI_2; *p<0.05 compared to the presence or absence of StHt31P; § p<0.05 basal compared to PGI_2/StHt31P **(C)** Washed platelets ($2.5x10^8$/ml) were pretreated with vehicle or RIAD-Arg_{11} (1, 10µM for 60minutes) then stimulated with (i) vWF/Ristocetin (10 µg/ml, 0.75mg/ml) or (ii) vWF/Ristocetin (20µg/ml, 0.75mg/ml). (iii) Washed platelets were

incubated with RIAD-Arg_{11} (10μM for 60minutes) or scrRIAD-Arg_{11} (10μM for 60minutes) alone and aggregation was evaluated. Shown are representative traces of three independent experiments.

Figure 6: RIAD-Arg_{11} modulates PGI_2-mediated inhibition of platelet arrest to vWF under conditions of flow.

Washed platelets were pre-incubated with either RIAD-Arg_{11} (10μM) or scrRIAD-Arg_{11} (10μM) for 60minutes followed by PGI_2 (100nM) for 1minute. Platelets were recombined with autologous RBC and the reconstituted blood was flowed over immobilised vWF (100μg/ml), for 4minutes at $1000s^{-1}$ and resulting platelet aggregates were viewed by fluorescent microscopy. (A) Representative images from 4 independent experiments and (B) % surface area coverage. Data are shown as % area coverage and are expressed as means ± SEM of 4 experiments. * $p<0.05$ and NS: not significant.

Figure 7: A model of the AKAP-dependent localisation of PKA-I into platelet lipid rafts and the resultant phosphorylation of GPIbβ. For clarity, GPIbα, GPV and GPXI have been omitted. In the top panel a pool of PKAI is localised to lipid rafts by the AKAP moesin facilitated the phosphorylation of GPIbβ. In the bottom panel the inclusion of the anchoring disruptor peptide, RIAD-Arg11, uncouples PKAI from its AKAP and thereby prevents phosphorylation of GPIbβ.