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## **Lighting up kinase action in platelets**

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In this issue of the Journal of Thrombosis and Haemostasis Hiratsuka et al. [1] present novel data on the roles of extracellular signal-regulated kinase (ERK) and cAMP-dependent protein kinase (PKA) during thrombus formation in mice.

Platelet functions are highly regulated by signaling networks involving at least 300 protein kinases [2]. Platelets express members of the mitogen-activated protein kinase (MAPK) family including p42 ERK2 and, at lower concentrations, p44 ERK1. Both ERK isoforms are activated by agonists like von Willebrand factor, collagen, thrombin, ADP and thromboxane A<sub>2</sub> [3-7]. ERK activation depends on MAPK kinase (MKK-1 or MEK) and involves G<sub>q</sub>, G<sub>i</sub>, phospholipase C and Src family kinase signaling [5, 8]. Active ERK has been shown to stimulate thromboxane A<sub>2</sub> production [5] and to contribute to platelet aggregation [4, 9]. Downstream substrates of ERK have not been identified in platelets so far.

PKA comprises a group of proteins all of which are expressed in human platelets including the regulatory subunits I $\alpha$ , I $\beta$ , II $\alpha$ , II $\beta$  and the catalytic subunits  $\alpha$ ,  $\beta$ ,  $\gamma$  [2]. PKA is activated by cAMP following G-protein  $\alpha$ -s (G<sub>s</sub>)-mediated stimulation of adenylate cyclase (AC). A key G<sub>s</sub>-coupled receptor in platelets is the IP-receptor for prostacyclin (prostaglandin I<sub>2</sub>, PGI<sub>2</sub>), a short-lived molecule released from healthy endothelial cells. Other G<sub>s</sub>-coupled receptors are A<sub>2A</sub> and A<sub>2B</sub> receptors for adenosine and vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor 1 [10]. G<sub>s</sub>-mediated AC activation is balanced by G<sub>i</sub>-mediated AC inhibition mainly through the G<sub>i</sub>-coupled P2Y<sub>12</sub> receptor for ADP. The cAMP/PKA pathway is known as strong inhibitory pathway and PKA activation has been shown to induce disaggregation of platelet aggregates [11-13]. PKA action involves the phosphorylation of a large number of substrate proteins [14, 15].

To study kinase activity in living cells sensor proteins based on Förster resonance energy transfer (FRET) have been developed [16, 17] and optimized [18, 19]. The structure of the sensors consists of consensus substrate phosphorylation sites for ERK (PDVPRTP,

phosphorylated residue underlined) or PKA (LLRRATL) as well as corresponding phosphorylation site binding domains which are separated from the phosphorylation sites by optimized linker domains. Phosphorylation of the threonine residues within the substrate sites by endogenous ERK or PKA leads to intramolecular interactions resulting in FRET between attached fluorescent proteins. These kinase sensors have been expressed and studied in transgenic mouse models before [20-22] and in the present study an analysis of the platelets of these animals is presented.

Initially Hiratsuka et al. [1] confirmed that sensor expression did not affect overall platelet functions as measured by flow cytometry of surface markers. FRET responses were then analysed in three experimental settings: isolated platelets adherent to collagen, isolated platelets perfused through collagen coated flow chambers, and in vivo thrombus formation after laser injury of subcutaneous arterioles. Platelets expressing the ERK sensor responded to thrombin treatment with an increased FRET signal, as expected. Blockage of MEK with PD0325901, a specific MEK inhibitor [23], abolished the thrombin-induced ERK signal. Flow chamber experiments indicated pronounced ERK activation during aggregate formation. In the laser injury model ERK activity was low during the early phase of thrombus initiation and increased strongly during the propagation and growth of the thrombus remaining high in the late stabilization phase. MEK inhibition with PD0325901 potently blocked thrombus initiation and reduced thrombus size when applied at later stages of thrombus development. These data indicate a more important role for ERK during tethering of platelets, aggregation and thrombus formation than currently appreciated.

Analysis of isolated PKA sensor expressing platelets confirmed that FRET could be triggered by activators of the cAMP pathway, but not by ADP, thrombin, or NO-donors. Surprisingly, PKA appeared to be stimulated during platelet aggregation in spite of the absence of endothelial cells in the flow chamber. Analysis of PKA function during thrombus growth in arterioles led

to further unexpected findings. During the propagation and stabilization phases of thrombus formation a clear activation of PKA was detectable throughout the thrombus. A particularly strong PKA signal was observed at the downstream side of the growing thrombus where platelets were seen to detach. PKA activation with a membrane-permeable cAMP analog, dbcAMP, before laser injury completely prevented thrombus initiation similar to MEK inhibition. However, dbcAMP was not able to dissolve the established thrombus in spite of inducing increased signals by the PKA sensor. This inability of dbcAMP to dissolve the aggregate coincided with a lack of inhibition of ERK activation.

Some of these observations challenge current concepts of PKA function and regulation. The prevention of thrombus initiation in arterioles by dbcAMP is compatible with the established inhibitory role of the cAMP/PKA in platelets. PKA activation at the downstream shell of the thrombus concomitant with platelet detachment matches the capacity of the cAMP pathway to trigger disaggregation in the *in vitro* setting [11-13]. In a previous study of thrombus development after vascular injury constitutively active  $G_i$  signaling was associated with increased size of the thrombus shell and inhibition of the  $G_i$ -coupled  $P2Y_{12}$  receptor was shown to reduce the shell area [24]. These data indicate that the cAMP/PKA pathway might be able to not only prevent thrombus formation but also to limit thrombus growth. However, the FRET studies presented by Hiratsuka et al. [1] present evidence of PKA activity throughout the entire thrombus including the core area around the site of vascular injury. Interestingly, it was recently shown that  $P2Y_{12}$ -dependent  $G_i$  signaling contributes to the development of a tight seal in the core area of the thrombus which prevents extravascular protein accumulation [25].  $G_i$ -mediated inhibition of cAMP production might be required to balance PKA activation in the thrombus core. These observations suggest that activating and inhibitory pathways might, at least to some degree, be active simultaneously during thrombus development. The lack of effect of cAMP-analogs, which activate PKA directly, on ERK function and thrombus dissolution cannot be

attributed to Gi-mediated reduction of cAMP production. Therefore, it remains unclear why dbcAMP, in contrast to PD0325901, is not able to dislodge the thrombus. Regarding the mechanisms of PKA activation during thrombus formation a number of possibilities can be envisaged. Since PKA is usually thought to depend on the short-lived endothelial mediator PGI<sub>2</sub>, endothelial PGI<sub>2</sub> might contribute to PKA activation in the thrombus shell. However, it is difficult to imagine how PGI<sub>2</sub> would be able to penetrate into the thrombus core. Furthermore, PKA activation was also seen in the flow chamber setting in the absence of endothelial cells and thus, presumably, also of PGI<sub>2</sub>. These observations might indicate PGI<sub>2</sub>-independent activation of cAMP signaling. Possible mechanisms include other, currently unknown, IP-receptor ligands released by platelets, a role for adenosine receptors, inhibition of phosphodiesterase-mediated cAMP degradation in the presence of high basal rates of AC, or new ways of AC or PKA activation. Of note, an alternative cAMP-independent pathway of PKA activation has been described based on thrombin-induced detachment of the catalytic subunit of PKA from a NFκB complex [26].

Possible limitations of the present study include the specificity of the biosensor constructs. Given the fact that only short substrate peptides were used as kinase targets it cannot be ruled out that kinases other than ERK or PKA might contribute to sensor phosphorylation and FRET activity. Moreover, the subcellular localization of the sensors and their accessibility to kinases and phosphatases could affect the signals obtained. For example, PKA is known to be attached to anchoring proteins resulting in compartmentalized PKA signaling [27]. Another issue is the resolution of current imaging methods. It was not possible to clearly identify single platelets in the laser injury studies of the present paper. Since the sensors have to be studied in separate models it is currently also not possible to establish if ERK and PKA activation actually concurred in the same platelets at the same time. Specific markers might be helpful to distinguish between core and shell structures of the developing thrombus in future studies [24].

Nevertheless, integrating FRET sensors into platelet studies represents an important methodological and conceptual advance. The pronounced effects of the MEK inhibitor might indicate MEK/ERK as new targets for anti-platelet therapy. The observed involvement of PKA not only in thrombus prevention but also during thrombus formation is likely to stimulate further research on the role of cAMP/PKA signaling in haemostasis and thrombosis.

## **Addendum**

A. Smolenski wrote the manuscript.

## **References**

- 1 Hiratsuka T, Sano T, Kato H, Komatsu N, Imajo M, Kamioka Y, Sumiyama K, Banno F, Mijata T, Matsuda M. Live imaging of ERK and PKA activities during thrombus formation in mice expressing biosensors based on Förster resonance energy transfer. *Journal of Thrombosis and Haemostasis*. 2017.
- 2 Burkhart JM, Vaudel M, Gambaryan S, Radau S, Walter U, Martens L, Geiger J, Sickmann A, Zahedi RP. The first comprehensive and quantitative analysis of human platelet protein composition allows the comparative analysis of structural and functional pathways. *Blood*. 2012; **120**: e73-82.
- 3 Gambaryan S, Geiger J, Schwarz UR, Butt E, Begonja A, Obergfell A, Walter U. Potent inhibition of human platelets by cGMP analogs independent of cGMP-dependent protein kinase. *Blood*. 2004; **103**: 2593-600.

- 4 Garcia A, Quinton TM, Dorsam RT, Kunapuli SP. Src family kinase-mediated and Erk-mediated thromboxane A<sub>2</sub> generation are essential for VWF/GPIb-induced fibrinogen receptor activation in human platelets. *Blood*. 2005; **106**: 3410-4.
- 5 Garcia A, Shankar H, Murugappan S, Kim S, Kunapuli SP. Regulation and functional consequences of ADP receptor-mediated ERK2 activation in platelets. *Biochem J*. 2007; **404**: 299-308.
- 6 Oury C, Toth-Zsamboki E, Vermeylen J, Hoylaerts MF. P2X(1)-mediated activation of extracellular signal-regulated kinase 2 contributes to platelet secretion and aggregation induced by collagen. *Blood*. 2002; **100**: 2499-505.
- 7 Tulasne D, Bori T, Watson SP. Regulation of RAS in human platelets. Evidence that activation of RAS is not sufficient to lead to ERK1-2 phosphorylation. *Eur J Biochem*. 2002; **269**: 1511-7.
- 8 Falker K, Lange D, Presek P. ADP secretion and subsequent P2Y<sub>12</sub> receptor signalling play a crucial role in thrombin-induced ERK2 activation in human platelets. *Thromb Haemost*. 2004; **92**: 114-23.
- 9 Moroi AJ, Watson SP. Akt and mitogen-activated protein kinase enhance C-type lectin-like receptor 2-mediated platelet activation by inhibition of glycogen synthase kinase 3 $\alpha$ /beta. *J Thromb Haemost*. 2015; **13**: 1139-50.
- 10 Smolenski A. Novel roles of cAMP/cGMP-dependent signaling in platelets. *J Thromb Haemost*. 2012; **10**: 167-76.



- 11 Kikura M, Kazama T, Ikeda T, Sato S. Disaggregatory effects of prostaglandin E1, amrinone and milrinone on platelet aggregation in human whole blood. *Platelets*. 2000; **11**: 446-58.
- 12 Stamler JS, Vaughan DE, Loscalzo J. Synergistic disaggregation of platelets by tissue-type plasminogen activator, prostaglandin E1, and nitroglycerin. *Circ Res*. 1989; **65**: 796-804.
- 13 White JG, Rao GH. Aggregated-disaggregated, refractory platelets retain sensitivity to ristocetin. *Thromb Res*. 1996; **84**: 253-66.
- 14 Beck F, Geiger J, Gambaryan S, Solari FA, Dell'Aica M, Lorocho S, Mattheij NJ, Mindukshev I, Potz O, Jurk K, Burkhart JM, Fufezan C, Heemskerk JW, Walter U, Zahedi RP, Sickmann A. Temporal quantitative phosphoproteomics of ADP stimulation reveals novel central nodes in platelet activation and inhibition. *Blood*. 2017; **129**: e1-e12.
- 15 Beck F, Geiger J, Gambaryan S, Veit J, Vaudel M, Nollau P, Kohlbacher O, Martens L, Walter U, Sickmann A, Zahedi RP. Time-resolved characterization of cAMP/PKA-dependent signaling reveals that platelet inhibition is a concerted process involving multiple signaling pathways. *Blood*. 2014; **123**: e1-e10.
- 16 Harvey CD, Ehrhardt AG, Cellurale C, Zhong H, Yasuda R, Davis RJ, Svoboda K. A genetically encoded fluorescent sensor of ERK activity. *Proc Natl Acad Sci U S A*. 2008; **105**: 19264-9.

- 17 Zhang J, Ma Y, Taylor SS, Tsien RY. Genetically encoded reporters of protein kinase A activity reveal impact of substrate tethering. *Proc Natl Acad Sci U S A*. 2001; **98**: 14997-5002.
- 18 Allen MD, Zhang J. Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem Biophys Res Commun*. 2006; **348**: 716-21.
- 19 Komatsu N, Aoki K, Yamada M, Yukinaga H, Fujita Y, Kamioka Y, Matsuda M. Development of an optimized backbone of FRET biosensors for kinases and GTPases. *Mol Biol Cell*. 2011; **22**: 4647-56.
- 20 Hiratsuka T, Fujita Y, Naoki H, Aoki K, Kamioka Y, Matsuda M. Intercellular propagation of extracellular signal-regulated kinase activation revealed by in vivo imaging of mouse skin. *Elife*. 2015; **4**: e05178.
- 21 Kamioka Y, Sumiyama K, Mizuno R, Sakai Y, Hirata E, Kiyokawa E, Matsuda M. Live imaging of protein kinase activities in transgenic mice expressing FRET biosensors. *Cell Struct Funct*. 2012; **37**: 65-73.
- 22 Mizuno R, Kamioka Y, Kabashima K, Imajo M, Sumiyama K, Nakasho E, Ito T, Hamazaki Y, Okuchi Y, Sakai Y, Kiyokawa E, Matsuda M. In vivo imaging reveals PKA regulation of ERK activity during neutrophil recruitment to inflamed intestines. *J Exp Med*. 2014; **211**: 1123-36.

- 23 Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P. The selectivity of protein kinase inhibitors: a further update. *Biochem J*. 2007; **408**: 297-315.
- 24 Stalker TJ, Traxler EA, Wu J, Wannemacher KM, Cermignano SL, Voronov R, Diamond SL, Brass LF. Hierarchical organization in the hemostatic response and its relationship to the platelet-signaling network. *Blood*. 2013; **121**: 1875-85.
- 25 Welsh JD, Muthard RW, Stalker TJ, Taliaferro JP, Diamond SL, Brass LF. A systems approach to hemostasis: 4. How hemostatic thrombi limit the loss of plasma-borne molecules from the microvasculature. *Blood*. 2016; **127**: 1598-605.
- 26 Gambaryan S, Kobsar A, Rukoyatkina N, Herterich S, Geiger J, Smolenski A, Lohmann SM, Walter U. Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFkappaB-IkappaB complex. *J Biol Chem*. 2010; **285**: 18352-63.
- 27 Raslan Z, Aburima A, Naseem KM. The Spatiotemporal Regulation of cAMP Signaling in Blood Platelets-Old Friends and New Players. *Front Pharmacol*. 2015; **6**: 266.