Platelet myosin light chain phosphatase: keeping it together

Ahmed Aburima*[1] and Khalid M. Naseem*

*Thrombosis Research Laboratory, Centre for Cardiovascular and Metabolic Research, Hull York Medical School, Lab 013/014, Hardy Building, University of Hull, Cottingham Road, Hull HU6 7RX, U.K.

Abstract

MLCP (myosin light chain phosphatase) regulates platelet function through its ability to control myosin IIa phosphorylation. Recent evidence suggests that MLCP is a *de facto* target for signalling events stimulated by cAMP. In the present mini-review, we discuss the mechanisms by which cAMP signalling maintains MLCP in an active state to control platelet contractile machinery.

Introduction

Circulating blood platelets scan the integrity of the vascular endothelium for damage and adhere to areas of injury where the extracellular matrix proteins collagen and vWF (von Willebrand factor) have become exposed. These adhesive proteins in combination with soluble platelet agonists, ADP, TxA_2 (thromboxane A_2) and thrombin trap and activate platelets to ensure the rapid formation of a haemostatic plug. The adhesion and activation of blood platelets at sites of vascular injury is essential for haemostasis, but, if unregulated, can also initiate thrombosis. To control the haemostatic response the endothelium releases PGI_2 (prostaglandin I_2, also known as prostacyclin), a potent platelet inhibitor. The marginalization of platelets in the blood vessel ensures that they are bathed in PGI_2 as they pass through the circulation. PGI_2 and other prostanoids such as PGE_1 (prostaglandin E_1) inhibit platelet function through a cAMP-dependent signalling cascade. Increased cAMP levels activates PKA (cAMP-dependent protein kinase), the primary effector of cAMP signalling in platelets, which blunts multiple aspects of platelet function through phosphorylation of numerous target proteins [1]. It is likely that, in healthy blood vessels, platelets are in a state of elevated cAMP, which acts to set the threshold for platelet activation. The cAMP signalling pathway controls multiple aspects of platelet function including Ca^{2+} mobilization, dense granule secretion and integrin αIIbβ3 activation and aggregation [2–4], although the molecular mechanisms underpinning the control of these functional responses are poorly understood. By defining the precise signalling events by which cAMP signalling regulates independent aspects of platelet function, we can develop a greater understanding of platelet function in health and disease. In the present mini-review, we explore how one aspect of platelet function, shape change, is regulated by cAMP signalling.

Platelet shape change

Platelet adhesion at sites of vascular damage stimulates a sequence of signalling events that cause dramatic morphological changes leading to generation of filopodia and lamellipodia [5]. These events are driven by a dynamic remodelling of their actin cytoskeleton, a complex process driven by both signalling events, which regulate actin dynamics, and proteins that bind actin directly to facilitate its polymerization [6]. Myosin IIa, an ATPase motor protein, interacts with actin to form a contractile unit whose function is analogous to that of actomyosin in smooth muscle cells. Myosin IIa is a hexamer composed of two heavy chains and four light chains. Phosphorylation of MLC (myosin light chain) on Ser^{19} endows myosin ATPase activity that facilitates the contraction of actin filaments and is therefore critical to shape change. The phosphorylation state of MLC is determined by the activities of two critical regulatory enzymes, MLCK (MLC kinase) and MLCP (MLC phosphatase). MLCK phosphorylates myosin at Ser^{19}, whereas MLCP dephosphorylates the same residue. In vascular smooth muscle cells, MLCP is a critical signalling hub that regulates the contractile state of the cells. Inhibitory phosphorylation of MLCP results in an increased Ca^{2+}-sensitivity and a contractile phenotype [7]. Similarly, platelet agonists such as thrombin and TxA_2 stimulate co-ordinated signalling events through GPCRs (G-protein-coupled receptors) that lead to simultaneous activation of MLCK and inhibition of MLCP [8,9], which collectively promote maximal MLC phosphorylation, driving both shape change and secretion of platelet granules [10,11]. Platelets and vascular smooth muscle cells contain identical isoforms of MLCP [12], although the role and regulation of MLCP in platelets is poorly elaborated.

MLCP is a heterotrimer that consists of a 37 kDa catalytic subunit (PP1cδ), a 110 kDa regulatory subunit termed MYPT1 (myosin phosphatase targeting subunit 1) and a 20 kDa protein (M20) of unknown function [13]. PP1c is

Key words: cAMP, myosin light chain phosphatase (MLCP), platelet, protein kinase A (PKA), RhoA, Rho-associated kinase (ROCK).

Abbreviations: GDI, guanine-nucleotide-dissociation inhibitor; GPCR, G-protein-coupled receptor; MLC, myosin light chain; MLCK, MLC kinase; MLCP, MLC phosphatase; MYPT1, myosin phosphatase targeting subunit 1; PG, prostaglandin; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; ROCK, Rho-associated kinase; TxA_2, thromboxane A_2.

[1]To whom correspondence should be addressed (email a.aburima@hull.ac.uk).

a family of phosphatases that exhibit a high degree of homology, but are proposed to fulfil non-redundant biological functions. The activity of individual isoforms are focused to specific substrates through their association with targeting subunits [14]. MYPT1 binds PP1cδ via its N-terminal RVXF (Arg-Val-Xaa-Phe) ankyrin repeats [15], whereas the C-terminus of MYPT1 binds myosin to focus the phosphatase close to its substrate [16]. The phosphorylation status of MYPT1 also has a major impact on determining the activity of MLCP. The targeting subunit is regulated by multiple protein kinases including ROCK (Rho-associated kinase), PKA and PKG (cGMP-dependent protein kinase) and indirectly by isoforms of PKC (protein kinase C). To date, five phosphorylation sites, i.e. Ser^{692}, Ser^{695}, Thr^{696}, Ser^{852} and Thr^{853}, have been identified on MYPT1. The three best-characterized phosphorylation sites are Ser^{695}, Thr^{696} and Thr^{853}, and current understanding suggests that the phosphorylation of these sites is a major determinant of MLCP activity. Phosphorylation of Thr^{696} and Thr^{853} is associated with reduced enzyme activity. In contrast, phospho-MLCP-Ser^{695} leads to increased activity possibly by relieving inhibitory phosphorylation [17]. The regulation of this key signalling node in platelets is less clear, but recent data aimed at dissecting the signalling events that modulate these phosphorylation sites have emerged to aid our understanding of how this critical enzyme is controlled and how it in turn influences platelet function.

The inhibition of MLCP activity drives platelet shape change

Platelet shape change requires inhibition of MLCP to facilitate actin contraction. The inhibition of MLCP activity is mediated primarily through RhoA and ROCK. Platelet activation induces the conversion of RhoA from its inactive GDP-bound form into its active GTP-bound form. The GTP–GDP cycle is tightly regulated by GEFs (guanine-nucleotide-exchange factors), GAPs (GTPase-activating proteins) and GDIs (guanine-nucleotide-dissociation inhibitors) (reviewed in [18]). RhoA is activated by numerous platelet agonists and is critical to shape change, stress fibre formation in spread platelets and stable thrombus formation under high shear [19,20]. In the active GTP-bound form, RhoA is translocated to the cell membrane via its readily prenylated CAAX (Cys-Ala-Ala-Xaa)-box at the C-terminus [21], where it binds and activates its downstream effector ROCK [22]. Although there are two isoforms of ROCK in platelets [23], their isoform-specific functions are unclear. The activation of ROCK by platelet agonists leads to MLC phosphorylation [19]. A more detailed analysis of this event demonstrated that platelet shape change and MLC phosphorylation required both Ca^{2+}-mediated activation of MLCK and ROCK-mediated inhibition of MLCP [24]. We found that thrombin induces phosphorylation of Thr^{696} (A. Aburima and K.M. Naseem, unpublished work) and Thr^{853} of MYPT1 in a ROCK-dependent manner to reduce MLCP activity [23]. Integrin-linked kinase also phosphorylates these inhibitory sites in platelets, although the physiological importance of these events are less clear [25,26]. The inhibitory phosphorylation of MYPT1 is facilitated by the formation of a tertiary signalling complex between RhoA, ROCK and MYPT1 [8]. More recently, we showed that, whereas both ROCK1 and ROCK2 are basally associated with RhoA in platelets, thrombin-mediated platelet activation led to the formation of a RhoA–ROCK2–MYPT1 complex from which ROCK1 was excluded [23]. These data suggest that phosphorylation of platelet MYPT1 is a ROCK2-mediated event, and potentially, that RhoA–ROCK1 and RhoA–ROCK2 complexes could play distinct roles in platelet function, although more sophisticated pharmacological agents and genetic ablation studies will be required to establish this.

Although the phosphorylation of MLCP by ROCK and its association with inhibition of MLCP activity is now established, the exact contribution of MYPT1 phosphorylation to the activity of MLCP is unclear. Khromov et al. [27] suggested that phosphorylation of MYPT1 converts the modified regions of the protein into an autoinhibitory domain that docks at the PP1cδ active site to inhibit activity. Alternatively, ROCK-mediated phosphorylation of MYPT1 blocks binding to myosin, which accounts for the reduction in MLCP activity [28]. In transfected COS7 cells, 14-3-3 protein dissociates MLCP from myosin II and attenuates MLCP activity [29]. In platelets, our data demonstrate that the phosphorylation of MYPT1 by ROCK leads to the dissociation of the PP1cδ catalytic subunit from its targeting subunit, thereby preventing its ability to localize with myosin IIa [30]. The ability of the active site of PP1cδ to bind to myosin IIa is also influenced by CPI-17 phosphoprotein. In smooth muscle cells, activation of PKC isoforms causes phosphorylation of CPI-17 Thr^{38} [31], which binds to and inhibits the MLCP catalytic subunit PP1cδ, possibly by acting as a pseudosubstrate. In contrast, platelet CPI-17 is constitutively associated with MLCP [32]. However, PKC-mediated phosphorylation of CPI-17 also results in the inhibition of MLCP activity [32]. Studies using microcystin, a hepatotoxin that selectively inhibits PP1c, show that CPI-17 docks directly at the PP1cδ active site and suppress its activity [27]. The mechanism by which phosphorylation of CPI-17 induces the inhibition of platelet MLCP is unclear and requires clarification. Thus MLCP activity is modulated by various mechanisms including dissociation of the holoenzyme subunits [33], phosphorylation of MYPT1 subunit by ROCK [34] and the binding of phospho-CPI-17 to the catalytic subunit [35].

Regulation of MLCP activity by cyclic nucleotide signalling

It has been recognized for many years that cyclic nucleotide signalling can regulate cellular contractile machinery [1]. Proteomic studies have shown that as many as 26 proteins associated with actin cytoskeletal organization are potentially regulated by PKA [36]. Signalling events downstream of cAMP inhibit MLC phosphorylation in platelets, endothelial cells and smooth muscle cells [37]. Consistent with

these observations, PGI_2 can inhibit platelet shape change either in suspension or when spreading on an adhesive surface [38], suggesting that cAMP signalling regulates platelet cytoskeletal reorganization. Since cAMP prevents the elevation of intracellular Ca^{2+} in platelets, it was assumed that inhibition of Ca^{2+}-dependent MLCK was the key site of regulation [2]. However, the demonstration that platelet shape change and MLC phosphorylation occurred through Ca^{2+}-dependent and -independent mechanisms [24] suggested the possibility that cAMP signalling may control actin remodelling independently of effects on Ca^{2+} mobilization. There is now significant evidence to suggest that cAMP targets the pathway leading to the inhibition of MLCP. By regulating several elements of this pathway, cAMP signalling effectively 'disinhibits' MLCP, allowing the phosphatase to control the extent of MLC phosphorylation. The evidence to support this hypothesis is outlined below.

Targeting of platelet receptors

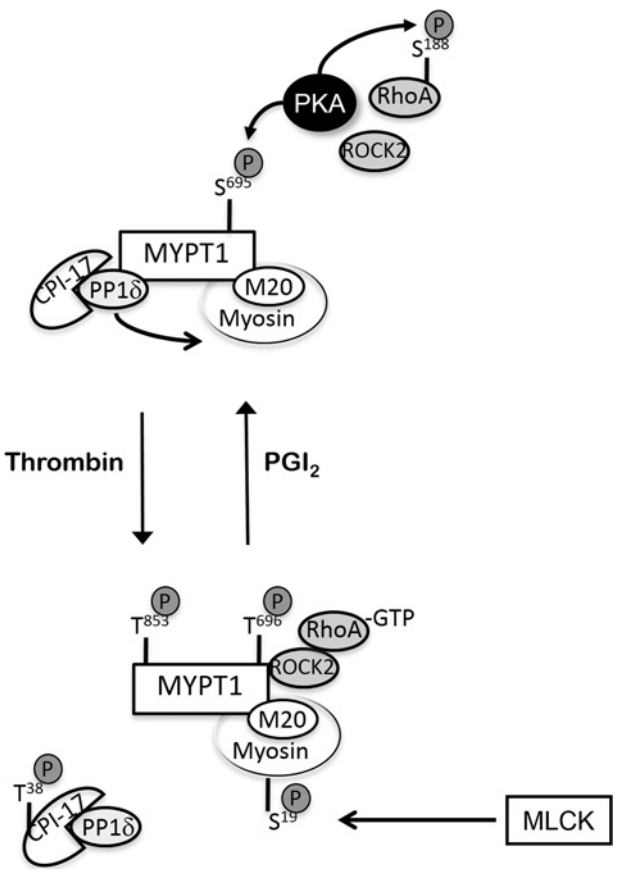
Platelet stimulation through GPCRs linked to $G\alpha_{12/13}$ leads to the activation of RhoA–ROCK. Several strands of evidence suggest that this early part of the pathway is targeted by cAMP signalling. The TxA_2 receptor, $TP\alpha$, is expressed in human platelets and studies in HEK (human embryonic kidney)-293 cells have suggested that this receptor is phosphorylated by PKA in response to PGI_2, leading to its desensitization [39]. Although receptor desensitization has not been demonstrated directly in platelets, it would clearly reduce signalling events that lead to inhibition of MLCP. *In vitro* studies demonstrate that PKA, activated by PGI_2 or by cAMP analogues, phosphorylated and inhibited $G\alpha_{13}$ [40], leading to inhibition of RhoA activation [41]. Thus cAMP-mediated signalling has the capacity to prevent receptor-mediated activation of the pathway required for MLCP inhibition.

Control of RhoA GTPase

Downstream of $G\alpha_{12/13}$ lies RhoA–ROCK, and our recent studies have demonstrated that cAMP signalling inhibits RhoA and ROCK activation independently of any effects on $G\alpha_{12/13}$ [23]. We demonstrated that physiological activation of cAMP signalling with PGI_2 led to the phosphorylation of RhoA on Ser^{188} and inhibition of RhoA activation. Translocation of RhoA from the platelet cytosol to the membrane is required for its activation, and phosphorylation by PKA prevents membrane compartmentalization of RhoA [23]. In other cells, PKA-mediated phosphorylation of RhoA leads to stabilization of RhoA with Rho-GDI protein in a complex localized in the cytosol [42], although this has not been demonstrated in platelets. The inhibition of RhoA membrane localization by cAMP in platelets prevents the inhibitory phosphorylation of MYPT1 on Thr^{695} and Thr^{853}, consistent with an absence of ROCK activity. Indeed, the phosphorylation of RhoA downstream of cAMP prevents the formation of the RhoA–ROCK2 complex. Under these conditions, ROCK2 cannot associate with and phosphorylate MYPT1, thereby preventing inhibition of MLCP activity.

Figure 1 | Structure and activity of MLCP are controlled by cAMP

MLCP can exist in two different states depending on the physiological conditions. In healthy blood vessels, platelets are continually exposed to PGI_2, which ensures elevated cytoplasmic cAMP. Here, PKA phosphorylates and inhibits RhoA allowing the MLCP holoenzyme to remain intact and activated. This in turn allows MLCP to maintain myosin in a dephosphorylated state. Activation of platelets leads to the recruitment of active GTP-bound RhoA and ROCK to the MLCP holoenzyme, causing phosphorylation of MYPT1 and dissociation of PP1cδ. Simultaneously, PKC-mediated phospho-CPI-17 is dislodged along with PP1cδ to ensure that the catalytic subunit does not act promiscuously on other substrates.



Phosphorylation of MLCP

There is strong evidence that cGMP signalling can control MLCP activity through direct PKG phosphorylation of MYPT1 on Ser^{695} [17,43]. Phosphorylation of this site does not activate MLCP [17,27], but rather facilitates the disinhibition of MLCP by preventing agonist-mediated inhibitory phosphorylation on Thr^{696} [17] and Thr^{853} [23]. The evidence for a role for cAMP signalling on direct regulation of MLCP is less developed. However, results from our laboratory demonstrate that PGE_1-mediated activation of cAMP signalling leads to the phosphorylation of MYPT1 on Ser^{695} (Figure 1) and is associated with a reduced inhibitory phosphorylation of MYPT1 on Thr^{696} and Thr^{853} (A. Aburima and K.M. Naseem,

unpublished work). The precise relationship between PKA-mediated phosphorylation of MYPT1 is unclear, but its phosphorylation by PKA suggests that MLCP is a novel and uncharacterized target for cAMP signalling and will require clarification in the future.

Controlling the holoenzyme structure

Another potential mechanism for regulation of MLCP by cAMP signalling is controlling the stability of the holoenzyme. Early studies demonstrated that isolated PP1cδ has reduced phosphatase activity towards phosphorylated MLC when examined *in vitro* [44]. Consistent with this concept, data from our laboratory show that, following platelet activation by thrombin, PP1cδ is released from the holoenzyme, resulting in a significant depression in platelet PP1cδ activity and allowing maximum MLC phosphorylation [23]. This loss of activity was driven by the dissociation of PP1cδ from MYPT1, a phenomenon already reported in smooth muscle cells [45]. cAMP elevation caused a modest increase in basal PP1cδ activity, but completely reversed the inhibitory effects of thrombin on PP1cδ activity. Central to this finding was the ability of cAMP signalling to prevent dissociation of PP1cδ from MYPT1, maintaining the holoenzyme complex and phosphatase activity.

Inhibiting the phosphorylation of CPI-17

The targeting of CPI-17 is another potential regulatory mechanism for the control of MLCP by cAMP signalling. In endothelial cells, cAMP signalling abrogates thrombin-induced hyperpermeability through activation of MLCP, caused by an inhibition of CPI-17 phosphorylation [46]. The mechanism described suggests that PKA can cause the dephosphorylation of CPI-17, leading to its dissociation from the PP1cδ–MYPT1 complex and preserving phosphatase activity. The reason for the presence of such a mechanism in platelets is unclear, but could represent another cAMP-controlled signalling node for regulation of cell contraction.

Conclusions

There is clear evidence to demonstrate that cAMP signalling inhibits MLCP activity in platelets. We hypothesize that the tonic release of PGI_2 from the endothelium stimulates continuous generation of cAMP in platelets, which acts to maintain the MLCP holoenzyme and activity to prevent platelet shape change. At sites of vascular damage, soluble agonists acting through ROCK inhibit MLCP to facilitate platelet shape change and secretion. Understanding the cross-talk between the pathways that inhibit and activate platelet MLCP is required to further our understanding of how the key signalling node controls platelet function.

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