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Calpain-mediated regulation of platelet signaling pathways

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Abstract

Purpose of review—There is considerable interest in understanding the function and mechanism of calpains in platelet aggregation, spreading, and granular secretion pathways. Recent insights from the calpain-1 knockout platelets suggest a pivotal role of these cysteine proteases in the regulation of outside-in signaling, aggregation, and clot retraction.

Recent findings—The calpain-1 knockout mouse provided direct evidence for the role of calpain-1 in platelet aggregation and clot retraction. Reduced tyrosine phosphorylation of platelet proteins correlated with reduced platelet aggregation and clot retraction. Future investigations of the mechanism of platelet defects in calpain-1 null mice may unveil the physiological functions of this important and elusive protease in mammalian cells.

Summary—This review focuses on the role of calpains in platelets with a particular emphasis on recent findings in calpain-1 null platelets. Previous studies used synthetic inhibitors to study the role of calpains in platelet function yielding useful information about the identification of calpain substrates. The development of calpain-1 null mice demonstrated that calpain-1 plays an important function in the regulation of platelet aggregation and clot retraction. Since the combined deletion of calpain-1 and calpain-2 genes results in embryonic lethality, the calpain-1 null mouse remains the only experimental model available to study the physiological role of calpains in mammalian cells.

Keywords

aggregation; calpain-1; protein tyrosine kinases; protein tyrosine phosphatases

Introduction

Traditionally, the calpain protease system consists of two calcium-dependent cysteine proteases called calpain-1 (μ -calpain) and calpain-2 (m-calpain), as well as calpastatin, an endogenous inhibitor of the two calpains [1]. Calpain-1 and calpain-2 enzymes are designated as conventional calpains based on their homology to the calcium-binding calmodulin-like domain in their catalytic subunits. Both calpains are heterodimers composed of an identical 28 kDa regulatory subunit and distinct 80 kDa catalytic subunits. The regulatory subunit (28 kDa) is encoded by the *Capn4* gene whereas the catalytic subunits (80 kDa) of calpain-1 and calpain-2 are the products of two independent genes, *Capn1* and *Capn2*, respectively. Recent cloning and sequencing studies have identified 12 additional mRNAs in mammals that encode polypeptides homologous to domains IIB and IIA of the 80 kDa subunits of two calpains [1,2]. The calpain-like mRNAs have also been identified in some lower organisms [3]. The polypeptides encoded by most of these calpain-like mRNAs,

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however, have not been isolated and therefore their in-vivo biochemical properties and functions remain unknown [1]. Both calpain-1 and calpain-2 are found in human platelets and the existence of their murine counterparts with 88% sequence identity suggests a conserved function across species. In mouse platelets, approximately 80% of the cysteine protease activity is contributed by calpain-1, whereas calpain-2 accounts for the remaining 20% protease activity. Calpain-1 is activated at micromolar calcium concentration, whereas calpain-2 requires millimolar calcium for its activation *in vitro*. It is believed that calpains can be activated by a variety of signals emanating from integrins to G-protein coupled receptors (GPCRs) that lead to the mobilization of calcium upon platelet activation.

Calpain-dependent regulation of integrin and G-protein coupled receptor signaling

Calpains have been implicated in the modulation of integrin signaling, particularly the $\alpha_{IIb}\beta_3$ (GPIIb–IIIa) integrin-mediated outside-in signaling during platelet activation. Although calpains can be directly activated by the G-protein coupled thrombin receptors in human platelets [4,5], the requirement of $\alpha_{IIb}\beta_3$ integrin engagement for calpain activation has been well documented for soluble ligands [6] and adhesive ligands such as von Willebrand factor (vWF) [7]. Furthermore, these studies suggested that signals originating from multiple platelet receptors converge and couple to calpain activation upon agonist-induced platelet activation. The susceptibility of β_3 integrin cytoplasmic domain to proteolysis by calpains also implies a direct physical interaction of cysteine proteases with the integrin $\alpha_{IIb}\beta_3$ receptor [8,9]. Du *et al.* [8] identified four calpain cleavage sites flanking the two NXXY motifs in the integrin β_3 cytoplasmic domain of platelets. Integrin $\alpha_{IIb}\beta_3$ -mediated signaling generally results in the modification of the platelet cytoskeleton, thus affecting cell spreading, aggregation, and clot retraction processes. Consistent with these observations, the use of synthetic inhibitors of calpains such as calpeptin and MDL 28170 led to the identification of calpain substrates that are mostly constituents of the platelet cytoskeleton. Some of these calpain substrates include spectrin [10], adducin [11], talin, filamin (ABP) [12], vinculin [13], cortactin [14], myosin-light chain kinase [15], and dystrophin-related protein [16]. The utilization of synthetic cysteine protease inhibitors including MDL 28170 and calpastat led to the suggestion that calpains regulate physiological functions of platelets such as aggregation, secretion, and spreading [4]. This suggestion was consistent with the evidence that the limited proteolytic modification of the cytoskeleton-associated substrates by calpains can trigger cytoskeletal reorganization in the target cells [17].

The first direct evidence for a functional role of calpains in platelets came from the generation of calpain-1 knockout mice, which show a reduced platelet aggregation and clot retraction phenotype [18]. More recent results demonstrate a key role of calpain-1 in the regulation of tyrosine phosphorylation of platelet proteins including the β_3 cytoplasmic domain. Calpain-1 null platelets are defective in platelet aggregation in response to a number of platelet agonists such as thrombin, collagen, ADP, and calcium ionophore A23187. Surprisingly, the proteolysis of talin, ABP, and β_3 integrin was not inhibited in the calpain-1 null platelets, suggesting that either calpain-2 can compensate for calpain-1 under these conditions or the calpain-1 enzyme does not mediate proteolysis of these substrates *in vivo*. It is noteworthy here that the expression of calpain-2 remains unaltered in the calpain-1-null mice. Interestingly, the tyrosine phosphorylation of multiple platelet proteins, including the β_3 integrin cytoplasmic domain, was reduced in the calpain-1-null platelets. The reduction of tyrosine phosphorylation correlated with the reduced platelet aggregation and clot retraction phenotype in the calpain-1-null mice. This observation is also consistent with the reduced platelet aggregation and clot retraction phenotype reported in the diYF mice, in which the two tyrosine residues of β_3 integrin cytoplasmic domain at positions 747

and 759 were replaced with phenylalanine by gene replacement technology [19]. Together, these studies further demonstrated the importance of tyrosine phosphorylation of $\alpha_{IIb}\beta_3$ integrin in mediating platelet outside-in signaling. The discovery that calpain-1 regulates platelet functions primarily by controlling the tyrosine phosphorylation of platelet proteins led to the identification of protein tyrosine phosphatase 1B (PTP1B, also called PTPN1), a major platelet tyrosine phosphatase, as a physiological substrate of calpains *in vivo* (our unpublished data). It is now becoming increasingly evident that calpains can modulate integrin-mediated platelet signaling by controlling the phosphorylation status of multiple proteins, presumably by regulating the activity of both tyrosine kinases and phosphatases [18,20–22].

The functional role of calpains in the attachment of $\alpha_{IIb}\beta_3$ integrin to the platelet cytoskeleton and in the regulation of clot retraction has been investigated before [23]. Since such studies relied upon the use of calpeptin and calpain inhibitor I, calpain inhibitors that also inhibit tyrosine phosphatases and other cysteine proteases, a conclusive role of calpains in integrin signaling and clot retraction pathways remained uncertain. The first definitive evidence for a functional role of calpain-1 in the clot retraction pathway was provided by the reduced clot retraction phenotype in the calpain-1 null mice, indicating an essential role of calpain-1 in the regulation of integrin–cytoskeletal interactions [18]. Another important aspect of platelet physiology is their ability to spread on immobilized ligands, thus sustaining normal hemostasis during wound healing and clot retraction events. Surprisingly, the mechanistic role of calpains has not been extensively investigated in platelet spreading despite the evidence that calpains are essential for cell migration and spreading processes in a variety of nucleated cells. For example, small G protein family members such as cdc42, Rac, and RhoA are needed for the formation of filipodia, lamellipodia, and stress fibers in cultured spreading cells. Interestingly, the calpain activity can modulate Rac and Rho A activation in nucleated cells [24,25]. Similarly, Vav, a well established activator of the small G protein family of GTPases, is a calpain substrate and has been implicated in platelet spreading [26]. The involvement of calpains in the spreading of thrombin-activated human platelets has been investigated using synthetic calpain inhibitors [4].

In nucleated cells, active calpains have been localized in the integrin clusters along with active Rac, however a requirement of calpain activity for focal adhesion and focal complexes induced by Rac and Rho A remains somewhat controversial [27–29]. The engagement of known GPCRs such as protease activated receptors (PARs), thromboxane A2 receptor (TPR), P2Y12, and P2Y1 leads to sustained calcium mobilization resulting in platelet shape changes and spreading [30–32]. Since the molecular mechanism of such spreading is currently unknown, a functional role of calpains in platelet spreading mediated by these GPCRs cannot be ruled out at this stage. The issue of the relative contribution of calpain-1 and calpain-2 in platelet spreading also remains unknown. Since the synthetic inhibitors of calpains lack specificity [33], the availability of a calpain-1 knockout mouse model with and without a mutation in the PTP1B gene provides a unique opportunity to test for the differential regulation of cdc42, Rac, and RhoA proteins by calpain-1 in the regulation of integrin and GPCRs during platelet spreading.

Role of calpains in granule secretion

Upon platelet activation, the granule secretion is a necessary step for sustaining the secondary wave of platelet aggregation and consolidation of platelet–platelet adhesion during clot retraction. Intracellular proteases, particularly calpains, have been implicated in the modulation of platelet secretory processes. For instance, the α -granule secretion in human platelets, as measured by P-selectin surface expression, was blocked by calpain inhibitors MDL 28170 and calpastat in response to SFLLR (thrombin peptide) and calcium

ionophore A23187 [4]. These findings implied that calpains could modulate early secretory events occurring within seconds of platelet activation. A functional role of calpains in the platelet secretory processes was also hinted from the membrane fusion studies. The membrane fusion machinery involves interactions between v-SNAREs, which are integral membrane proteins of the granules, and their counterparts present on the plasma membranes called t-SNAREs. Two independent studies have shown calpain-dependent proteolysis of SNAP-23, a t-SNARE, within 2–5 min of platelet activation [34,35]. The calpain inhibitors (calpeptin, calpastatin, calpain inhibitor IV, E-64d), however, failed to show any significant effect on platelet granule secretion in one of these studies [34]. In contrast, the treatment of calpain inhibitors resulted in the inhibition of α -granule secretion in permeabilized platelets [35]. Similarly, platelet VAMP-3, a v-SNARE, has been identified as a calpain substrate [35] but again the VAMP-3-null mouse platelets did not show any alterations in granule secretion [36]. Together, these studies reflect the complexities generally encountered by the utilization of synthetic inhibitors of calpains in platelet studies.

The availability of mouse knockout models lacking either calpains or their substrates provides a unique opportunity to evaluate the functional role of calpains in platelet secretion pathways. For example, in the calpain-1 knockout platelets, there was a moderate reduction in the thrombin and collagen-mediated dense granule secretion (ATP secretion) whereas activation by ADP and calcium ionophore A23187 did not show any effect on dense granule secretion [18]. The phospholipase C- β (PLC- β) and protein kinase C (PKC) have also been identified as calpain substrates, and could potentially modulate downstream signaling pathways of secretory processes in response to various agonists. A recent study [37] has demonstrated an impaired secretion of ATP in washed platelets from mice lacking both PLC β 2 and PLC β 3 (PLC β 2/3^{-/-}) in response to weak agonists such as ADP and thromboxane A₂ analog U46619 but not with collagen and thrombin [37]. The calpain-mediated proteolysis of PKC has been implicated in the regulation of PKC activity in a number of studies [38,39], and the treatment of platelets with PKC inhibitors enhances platelet secretion from the dense granules [40]. In contrast, the PKC δ knockout mice failed to show any defects in platelet secretion, indicating that the PKC inhibitors may inhibit multiple PKC isoforms *in vivo* [40]. Finally, the activation of calpains has been correlated with microvesicle release and procoagulant activity in platelets, further implicating these proteases in the platelet secretion pathways [41–43]. Using the newly developed mouse knockout models with a genetically defined background, future studies will be warranted to resolve a functional role of calpains in the differential regulation of platelet α , dense, and lysosomal granules. The presence of distinct vesicle pools containing PF4, P-selectin, vWF, and fibrinogen and their differential release by calpains remains an attractive hypothesis to be tested in future studies.

Calpain-regulated protein phosphorylation in platelets

A number of protein kinases such as Src, Syk, focal adhesion kinase (FAK), and PKC have been identified as calpain substrates in platelets [20,21,38,44]. Similarly, phosphatases such as SHP-1, PTP1B, inositol-4-phosphatase, and PTPMEG have been reported as calpain substrates [22,45–48]. These studies suggested that calpains may regulate the balance between kinase and phosphatase activities in platelets. Proteolytic degradation of pp^{60src} by calpains cleaves its amino-terminal membrane anchorage region, thus leading to the formation of truncated pp^{52src} and pp^{47src} polypeptides. In activated platelets, this limited proteolytic modification of Src causes a reduction in its tyrosine kinase activity as well as the relocation of truncated Src polypeptides into the cytosol, presumably resulting in the altered substrate specificity [20]. Similarly, proteolysis of focal adhesion kinase (pp^{125FAK}) by calpains results in the sequential generation of 90, 45, and 40 kDa forms of the enzyme, thus leading to its altered cellular distribution and reduced autokinase activity in activated

platelets [21]. In contrast, calpain-mediated proteolysis of FAK could not be confirmed in other studies under similar conditions [49,50]. The calpain activity has also been implicated in the amino-terminal cleavage of p^{72syk} into p^{52syk} and p^{45syk} polypeptides consistent with the notion that such proteolytic events may eventually lead to the inactivation of substrates in activated platelets [44]. Based on these studies, which were carried out using calpain inhibitors, the precise role of calpains in the modulation of platelet tyrosine kinases remains unresolved. It is noteworthy that the differential response of various tyrosine kinases to calpains could be a reflection of the level of calpain activation *in vivo*. For example, the treatment of platelets with calcium ionophore A23187 results in the full activation of calpains, whereas activation by physiological stimulants such as thrombin leads to only a partial activation of platelet calpains [21,45,51].

The functional role of calpains in the regulation of tyrosine phosphatases also remains poorly understood. The limited proteolysis of PTPMEG by calpains has been shown to enhance its phosphatase activity in platelets [48]. Calpain activity has also been investigated as a mechanism for the regulation of PTP1B, a major tyrosine phosphatase in platelets. PTP1B is believed to regulate tyrosine dephosphorylation of platelet proteins such as pp^{60Src} [52]. An earlier study demonstrated that calpains can rapidly cleave the carboxyl-terminus of full length 50 kDa PTP1B into a truncated 42 kDa form in calcium ionophore A23187 activated human platelets, and the cleaved 42 kDa form of PTP1B shows a two-fold increase in phosphatase activity compared with the full-length PTP1B [22]. It is important to note here, however, that this conclusion was based on experiments in which both 50 and 42 kDa forms of PTP1B were immunoprecipitated from calcium ionophore-activated human platelets, and their enzyme activity was measured using an immune complex phosphatase assay [22]. Since the 50 kDa form of PTP1B is known to be tightly associated with the internal membrane structures through its carboxyl-terminus and the truncated 42 kDa form of PTP1B is believed to be soluble, it is therefore likely that the surrounding milieu of the two immune complexes from platelets would not be the same. In fact, one can speculate that the detergent-solubilized membrane material likely to be associated with the immunoprecipitated full length 50 kDa form of PTP1B could be the source of enzyme inhibition. This mode of inhibition could in fact lead to the current perception that the calpain-cleaved 42 kDa form of PTP1B shows a two-fold increase in phosphatase activity compared with the full length 50 kDa PTP1B. A resolution of this key issue would require precise quantification of the specific activity of two forms of PTP1B under conditions in which both forms are soluble and their enzyme activity is not differentially influenced by factors such as phosphorylation and oxidation *in vitro*.

The emerging evidence in platelets as well as in other cells is consistent with the view that calpains are involved in the complete degradation of PTP1B, thus downregulating its phosphatase activity *in vivo*. For example, complete proteolysis of PTP1B in platelets that were activated for 5 min with 1 μ M calcium ionophore A23187 correlated with concomitant activation of calpains [41]. Another study showed complete degradation of PTP1B by calpains in response to UVA treatment of A431 cells [53]. Whether the truncated polypeptides of PTP1B generated during the course of calpain-mediated degradation retain tyrosine phosphatase activity and exhibit altered subcellular localization remains unknown. It is relevant to point out here that the susceptibility of another major tyrosine phosphatase, SHP-1, to calpain-mediated degradation is regulated by tyrosine phosphorylation. The calpains are known to cleave the amino-terminus of SHP-1 compared with the carboxyl-terminal cleavage of PTP1B. Thrombin-induced tyrosine phosphorylation of SHP-1 renders the phosphatase resistant to calpain-mediated proteolysis compared with its unphosphorylated form [45]. The notion that limited proteolysis of PTP1B and SHP-1 by calpains activates their phosphatase activity was further challenged with the demonstration that calpeptin, a calpain inhibitor, had no effect on the total phosphatase activity of calcium

ionophore-activated platelets [45]. Similarly, in the calpain-1 null mouse platelets, the tyrosine phosphorylation of several proteins is reduced, indicating that the limited cleavage of tyrosine phosphatases may not contribute to the activation of tyrosine phosphatases in mouse platelets. It is noteworthy here that the calpain-1 null mouse platelets still retain around 20% of total calpain activity due to the presence of calpain-2; yet the tyrosine phosphorylation of platelet proteins is significantly reduced in the mutant platelets. These observations are corroborated by the findings that the proteolysis of PTP α and PTP ϵ by calpains can also downregulate their phosphatase activity [54]. Although the presence of these receptor-type tyrosine phosphatases has not been demonstrated in mature platelets, the PTP α is known to regulate Src activity by causing dephosphorylation of tyrosine-527 in Src [54]. Another pertinent example is the downregulation of enzyme activity of polyphosphate-4-phosphatase upon its complete proteolysis by calpains in the calcium ionophore-activated platelets [47]. Together, these studies raise the possibility that calpain activity can both positively and negatively regulate the activity of tyrosine phosphatases under the given experimental conditions.

Other calpain substrates in platelets

Recently, a functional role of calpains has been proposed in the regulation of store-operated calcium entry in platelets [55]. Calpains have been implicated in the generation of the long form of β 3-endonexin [56], proteolysis of platelet endothelial cell adhesion molecule-1 [57], vinculin [13], plasma membrane Ca²⁺ ATPase [58], phosphatidylinositol phosphate kinase [59], and activation of calcineurin, a serine-threonine phosphatase [60]. The physiological significance of these findings remains to be explored in future studies.

Conclusion

Despite an intense interest in the function of calpains in platelets and other cells, their precise physiological role remains unknown. The use of synthetic inhibitors of calpains, which lack specificity, could be the origin of conflicting results reported for calpain functions in platelets. Studies on calpain-1 knockout platelets have revealed a major role of calpain-1 in the regulation of tyrosine dephosphorylation of proteins and its functional impact on the platelet cytoskeleton. The generation of calpain-1 and PTP1B double knockout mice, and the future development of conditional calpain-2 knockout mouse models, are potential opportunities that would finally allow resolution of the physiological functions of calpains *in vivo*.

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Abbreviations

FAK	focal adhesion kinase
GPCR	G-protein coupled receptor
PAR	protease activated receptor
PKC	protein kinase C
PTP1B	protein tyrosine phosphatase 1B
vWF	von Willebrand factor

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 298).

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