

Protease-activated receptor signaling: new roles and regulatory mechanisms

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Purpose of review

Protease-activated receptors are G-protein-coupled receptors that transmit cellular responses to coagulant proteases in a variety of cell types in the vasculature and other tissues. Several other proteases can activate protease-activated receptors *in vitro* and may affect their function *in vivo*. While a role for these receptors in hemostasis and thrombosis has been established, their functions in inflammatory and other responses have yet to be fully elucidated. In addition, the mechanisms responsible for protease and cell type-specific signaling mediated by these receptors are largely undefined. Here, we highlight recent advances in understanding the roles and regulation of protease-activated receptor signaling.

Recent findings

Recent studies have increased our knowledge of the function of protease-activated receptor signaling in platelets and its contribution to thrombosis. In other cell types, recent work has revealed new connections between these receptors and signaling effectors important for vascular development and inflammatory responses. Other studies have advanced our understanding of protease and cell type-specific responses as well as novel regulatory mechanisms for control of protease-activated receptor signaling.

Summary

Thus, elucidating the signaling and regulatory mechanisms of protease-activated receptors in various tissues and cell types is important for understanding their biological function as well as for designing therapeutic strategies to control their function.

Keywords

coagulant protease, G-protein, G-protein-coupled receptor, thrombin

Abbreviations

AP2	adaptor protein-2
APC	activated protein C
CNS	central nervous system
GPCR	G-protein-coupled receptor
PAR	protease-activated receptor
PKA	protein kinase A
PKC	protein kinase C
TRP	transient receptor potential

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Introduction

Protease-activated receptors (PARs) are G-protein-coupled receptors (GPCRs) that function in hemostasis and thrombosis, as well as in inflammatory and proliferative responses triggered by tissue injury. PARs signal in response to multiple extracellular proteases [1]. There are four PARs encoded in the mammalian genome. PAR1, the family prototype, transmits cellular responses to thrombin, the main effector protease of the coagulation cascade. In addition, PAR1 is activated by coagulation factor Xa, as well as by the anticoagulant proteases plasmin and activated protein C (APC). PAR3 and PAR4 signal mainly in response to thrombin, but PAR4 can also be activated by cathepsin G, a neutrophil secreted protease [2]. Multiple trypsin-like serine proteases activate PAR2 including trypsin, mast cell tryptase, factors VIIa and Xa but not thrombin. Several other proteases can activate PARs *in vitro* and may affect their function *in vivo*.

PARs are present in many cell types, with partially overlapping tissue expression patterns. PAR1, PAR3 and PAR4 are expressed primarily by vascular cells and are the major effectors of thrombin signaling *in vivo* [3]. PARs mediate thrombin signaling in platelet activation, which is critical for hemostasis and thrombosis. Activation of PARs on endothelial cells results in recruitment of immune cells, disruption of the endothelial cell barrier as well as migration and release of growth factors and cytokines. PAR2 is expressed by vascular, intestinal and airway cells and mediates inflammatory and proliferative responses associated with tissue injury. PAR1 and PAR2 are also expressed in sensory neurons and glial cells; the expression of PAR3 and PAR4 in these cell types is less clear. Activation of PARs in sensory neurons initiates neurogenic inflammation, edema and hyperalgesia. PAR activation in glial cells stimulates proliferation

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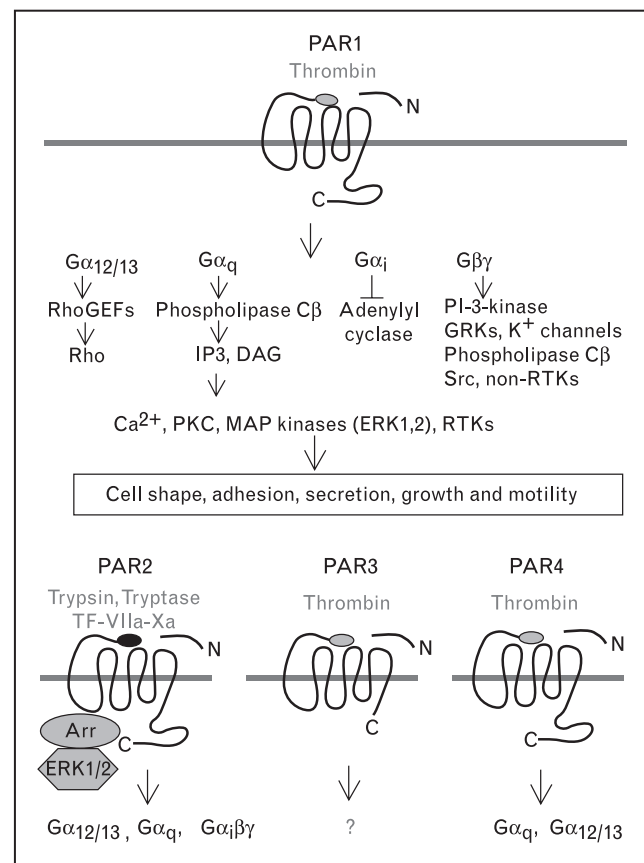
and release of neuroactive agents. While a role for PARs in hemostasis and thrombosis is clearly established, their functions in inflammatory and other responses have yet to be fully elucidated. In addition, the mechanisms responsible for protease and cell type-specific signaling mediated by PARs are just beginning to be defined. Here, we discuss recent advances in understanding PAR function and signaling in platelets, endothelial cells, neurons and glial cells.

Protease-activated receptor signaling and regulation

Proteases activate PARs by cleavage of an N-terminal peptide bond, which generates a new N-terminus that acts as a tethered ligand by binding intramolecularly to the receptor to trigger transmembrane signaling (Fig. 1). Once activated, PARs undergo conformational changes that facilitate coupling to heterotrimeric G proteins. PAR1 couples to G_{α_q} , G_{α_i} and $G_{\alpha_{12,13}}$ subtypes and induces activation of mitogen-activated protein (MAP) kinases, mobilization of intracellular Ca^{2+} and RhoGEF-mediated Rho and Rac signaling and other effectors to promote diverse cellular responses (Fig. 1) [3]. PAR4 also appears to couple to G_{α_q} and $G_{\alpha_{12,13}}$ but not G_{α_i} , at least in fibroblasts [4], whereas PAR3 has not been shown to signal through G proteins. Rather, mouse PAR3 acts as a cofactor that binds and localizes thrombin for activation of PAR4, a receptor that has a low affinity for thrombin [5]. Whether human PAR3 can signal by itself or together with other PARs remains to be determined. Although there is no direct evidence linking PAR2 to G-protein activation, numerous studies show that PAR2-selective agonists increase second messenger responses suggestive of G_{α_q} , G_{α_i} and perhaps $G_{\alpha_{12,13}}$ signaling (Fig. 1). In addition, activated PAR2 binds to and internalizes with arrestin, a multifunctional adaptor protein [6,7]. The interaction of internalized PAR2 with arrestin is thought to sustain extracellular regulated kinases-1,2 (ERK1,2) signaling in the cytoplasm independently of G-protein activation. PAR activation of distinct G-protein subtypes, as well as non-G-protein effectors such as arrestins are responsible for eliciting protease and cell type-specific responses.

The proteolytic activation of PARs is irreversible; thus, rapid desensitization and receptor trafficking tightly regulate PAR signaling. Desensitization of activated GPCRs occurs by phosphorylation and arrestin binding, which uncouples the receptor from G proteins. Arrestins also interact with clathrin and the clathrin adaptor protein-2 (AP2) to facilitate GPCR internalization. Once internalized, GPCRs are dephosphorylated, recycled back to the plasma membrane and competent to signal again. In fibroblasts and endothelial cells, activated PAR1 is rapidly desensitized by phosphorylation and arrestin binding, like most GPCRs [8–10]. Unlike most GPCRs,

Figure 1 Protease-activated receptor (PAR) signaling



PARs are G-protein-coupled receptors (GPCRs) that are activated by a unique proteolytic mechanism. Proteases activate PARs by cleaving an N-terminal peptide bond, generating a new N-terminus that acts as a tethered ligand and binds intramolecularly to the receptor to trigger transmembrane signaling. In most cases, synthetic peptides that mimic the tethered ligand domain can activate PARs independent of proteolysis. PAR1 couples to G_{α_q} , $G_{\alpha_{12,13}}$, G_{α_i} to activate a variety of signaling cascades and cellular responses. The $G_{\alpha_{12,13}}$ subunits bind RhoGEFs, which activate small G-proteins such as Rho. G_{α_q} activates phospholipase C- β which triggers phosphoinositide hydrolysis resulting in inositol triphosphate (IP $_3$) and diacylglycerol production, Ca^{2+} mobilization, protein kinase C (PKC), mitogen-activated protein (MAP) kinase activation as well as transactivation of receptor-tyrosine kinases (RTKs). $G_{\beta\gamma}$ can activate phosphatidylinositol 3 (PI-3) kinase, recruits G protein-coupled receptor kinases (GRKs) and other effectors. PAR2 also appears to signal to multiple G-protein subtypes in addition to non-G-protein effectors such as arrestins, that function as scaffolds to recruit and activate extracellular regulated kinases-1,2 (ERK 1,2) as well as other effectors. PAR4 signals to G_{α_q} and $G_{\alpha_{12,13}}$ but not G_{α_i} at least in certain cell types, whereas PAR3 has not yet been shown to couple to G-proteins.

activated PAR1 is internalized, sorted directly to lysosomes and degraded [11,12]. Internalization and lysosomal sorting of proteolytically activated PAR1 is thought to prevent the receptor from returning to the cell surface with its tethered ligand intact and continuing to signal [12,13]. Proteolysis or truncation of the tethered ligand may also occur to prevent further PAR1 signaling. Interestingly, activated PAR1 internalization does not

require arrestins and the internalization mechanism of activated PAR1 has yet to be defined [9,14]. Arrestins, however, do mediate desensitization and internalization of PAR2 [7], although phosphorylation of PAR2 has not been directly demonstrated. Internalization of activated PAR4 also appears to be a mechanism for signal termination but activated receptor phosphorylation has not been detected [15]. A slower internalization rate or lack of phosphorylation of activated PAR4 are thought to promote sustained signaling by this receptor [15,16]. The mechanisms that regulate cleaved PAR3 remain to be defined.

Protease-activated receptor function in platelets

Thrombin is generated at sites of vascular injury when tissue factor is exposed to plasma coagulant proteases. Tissue factor binds factor VII, supporting both its activation to VIIa and activation of factor X. Factor Xa acts with its cellular cofactor Va to generate thrombin, which drives fibrin deposition and platelet activation. Activated platelets also provide a surface for assembly of the tenase and prothrombinase complexes that produce more thrombin. Thrombin activation of platelets induces platelet shape change, release of ADP and thromboxane A₂ (TxA₂) and integrin-mediated aggregation. ADP and TxA₂ further act on platelets, creating a positive feedback loop to amplify platelet activation. PAR1 coupling to G α_q is necessary for platelet secretion and aggregation, G α_{13} contributes to shape change, and G $\alpha_{z/i}$ synergizes signaling by other platelet agonists, recently reviewed in [17]. In human platelets, activated PAR1 signaling is rapidly desensitized, in contrast to sustained PAR4 signaling [15,16]. Interestingly, activated PAR1 displays minimal internalization in human platelets [18]. Thus, the majority of cleaved PAR1 is retained on the platelet surface, suggesting that desensitization is sufficient to shut off PAR1 signaling.

The use of PAR knockout mice and blocking antibodies has been invaluable in establishing the roles of PARs in platelet activation and thrombosis. Platelets display species-specific differences in PAR expression. PAR3 and PAR4 mediate thrombin signaling in mouse platelets, whereas PAR1 and PAR4 are the functional thrombin receptors in human platelets. In human platelets, activation of PAR1 with low thrombin concentrations is sufficient to trigger secretion and aggregation [19,20]. By contrast, PAR4 mediates platelet activation at high thrombin concentrations in the absence of PAR1 function [21]. The contribution of PAR4 to normal human platelet activation by thrombin is not known. In mouse platelets, PAR3 does not signal but instead acts as a cofactor that facilitates thrombin cleavage and activation of PAR4 [5]. A series of elegant studies by Coughlin and coworkers have clearly shown a necessary role for PAR4 in thrombin

activation of mouse platelets and in thrombosis; see the recent review [3]. In new work, Vandendries and colleagues [22] further examined the role of PAR4 in laser-induced arteriole thrombosis in real-time using wildtype and *Par4*-deficient mice. The results from these studies reveal a critical role for PAR4 in propagation of the platelet thrombus away from the site of injury but not for juxtamural thrombus formation or fibrin deposition.

Protease-activated receptor activation and signaling in endothelial cells

Thrombin activation of endothelial cells triggers a host of cellular responses that contribute to hemostasis, inflammation and vascular development. PAR1 and PAR4 mediate thrombin signaling in mouse endothelial cells, whereas PAR1 and PAR3, but not PAR4, are the thrombin receptors present in human endothelial cells [23]. PAR1 function in endothelial cells is important for the formation and maintenance of blood vessels in embryonic development [24,25]. New studies indicate that G α_{13} may be a critical mediator of PAR1 signaling in endothelial cells during development [26]. Mouse embryos derived from endothelial cell-specific G α_{13} knockouts displayed a phenotype that resembles PAR1 deficiency and restoration of G α_{13} expression rescued this phenotype.

In addition to thrombin, the anticoagulant-protease APC can cleave and activate PAR1 on human endothelial cells [27], but whether PAR1 mediates APC responses *in vivo* is a subject of some controversy [28]. Protein C, the precursor of APC, is localized to the endothelial cell surface by binding to endothelial protein C receptor (EPCR) and cleaved and activated by thrombin bound to thrombomodulin. Although APC and thrombin are both thought to proteolytically activate PAR1 on endothelial cells, they promote anti-inflammatory and proinflammatory responses, respectively [29]. Intriguingly, low thrombin and high APC concentrations can reverse these opposing cellular responses, suggesting that the level of receptor activation is important for conferring specific cellular responses. The mechanistic basis for how activation of the same receptor by two different proteases can produce distinct cellular responses has not been determined. The compartmentalization of PARs and distinct G-protein subtypes in lipid rafts such as caveolae might confer PAR-G-protein selectivity and explain why APC bound to EPCR is markedly less efficient at stimulating G α_q -mediated responses but promotes ERK1,2 signaling comparable to that elicited by thrombin [29,30].

Unlike platelets, which presumably respond to thrombin only once, endothelial cells, fibroblasts and other cell types are exposed to thrombin repeatedly and need to recover thrombin signaling in a timely manner. This

appears to be accomplished by the movement of uncleaved PAR1 from an internal pool to the cell surface, which permits rapid recovery of thrombin signaling independent of de-novo receptor synthesis [31]. In recent work, Paing *et al.* [32**] demonstrate that the clathrin adaptor AP2 is essential for constitutive internalization of uncleaved PAR1 and cellular resensitization to thrombin signaling in endothelial cells and other cell types. The clathrin adaptor AP2 binds directly to a tyrosine-based motif in the cytoplasmic tail of PAR1. In cells expressing PAR1 tyrosine-based mutants or in cells depleted of AP2 by RNA interference, uncleaved PAR1 fails to constitutively internalize and to form an internal store, rendering cells unresponsive to additional thrombin stimulation. Whether disruption of PAR1 constitutive internalization affects other thrombin-stimulated endothelial cell responses will be important to determine.

PAR2 is also expressed in endothelial cells and can be activated by upstream coagulant factors VIIa and Xa [33,34]. VIIa activates PAR2 either directly in a binary complex with tissue factor or indirectly by generation of factor Xa, which can activate both PAR2 and PAR1. Xa may signal more efficiently in a ternary complex with TF:VIIa than it does as a monomer. Ahamed *et al.* [35**] recently found that binary signaling involves a distinct form of tissue factor similar to a cryptic form of tissue factor that is generated by disulfide bond cleavage mediated by protein disulfide isomerase. A tissue factor cysteine mutant displayed defective factor X activation and Xa signaling but was competent to support VIIa signaling through PAR2. The physiological function of binary TF:VIIa versus TF:VIIa:Xa signaling through PAR2 remains to be determined. It is possible that activation of PAR2 by TF:VIIa versus TF:VIIa:Xa elicits distinct cellular responses, perhaps by stabilizing distinct PAR2 conformations.

Protease-activated receptor signaling in neurons and astrocytes

PARs play important roles in the central nervous system (CNS), which may involve signaling pathways distinct from peripheral cells. Proteases capable of activating PARs are expressed in the CNS and PARs appear to signal in neurons and glia through multifunctional pathways [36]. Activation of PAR1 during neuropathological insult can promote neuronal injury or protection and affect neurite outgrowth. The signaling pathways linking PAR activation to these diverse tissue responses have not been fully elucidated [37].

Several recent studies have highlighted the importance of PAR2 in small sensory neurons of the autonomic ganglia. PAR2 activation has been implicated in neurogenic inflammation that involves Ca²⁺-dependent release of inflammatory mediators such as substance P and

calcitonin gene-related peptide from sensory neurons into peripheral tissue [38]. Intriguing recent work on neuronal PAR signaling has linked PAR2 to transient receptor potential (TRP) channel activation in sensory neurons. PAR2 colocalizes with TRPV1 in a subset of dorsal root ganglion neurons, and PAR2 activation lowers the threshold and potentiates signaling through capsaicin and heat-sensitive TRPV1 channels [39,40]. PAR2 activation in dorsal root ganglion cells promotes protein kinase-C ϵ (PKC ϵ) and A (PKA) translocation from the cytoplasm to the plasma membrane; PAR2 activation also increases TRPV1 phosphorylation [39]. Interestingly, PAR2 activation elevates cAMP in dorsal root ganglia neurons by an undetermined mechanism, consistent with the involvement of PKA in TRPV1 modulation [41*]. PKC ϵ and PKA inhibitors prevent PAR2 enhancement of TRPV1 function, and mutant TRPV1 channels that are insensitive to PKC phosphorylation are not influenced by PAR2. PAR2 activation and subsequent potentiation of TRP channel function exacerbates thermal hyperalgesia, which can be blocked by inhibition of PKC ϵ , suggesting that PAR2 potentiation of TRPV1 may hold therapeutic potential [39,40,41*]. In addition, PAR2 activation appears to mediate mechanical hyperalgesia through enhancement of TRP channel function [42]. TRPV4 is coexpressed with PAR2 in dorsal root ganglia neurons, and activation of PAR2 potentiates TRPV4 signaling by mechanisms involving PLC- β and PKC as well as PKA and PKD [42]. In contrast to PAR2, PAR1 and PAR4 appear to have antinociception effects in sensory neurons [43–45].

PARs activate multiple signaling pathways in astrocytes that have important consequences for normal brain function and for the response of CNS tissue to injury (reviewed in [37,46] and see [47,48] for discussion of microglial PARs). PAR1 expression is robust in human astrocytes [49]. Although recent studies suggest a role for PLC- β 3 in PAR1-mediated Ca²⁺ release in glial cells [50], the contribution of distinct G α subunits and PLC isoforms to PAR signaling in astrocytes is unknown. PAR1 is known to stimulate astrocyte proliferation *in vitro* and *in vivo*. PAR1 induces robust ERK1/2 activation, an important mediator of astrocyte proliferation. In contrast to transient ERK1,2 activation induced by many stimuli, activated PAR1 causes sustained ERK1/2 signaling in astrocytes [51]; see also [47]. Pharmacological studies suggest that sustained ERK1/2 activity is mediated in part by Rho kinase, and upregulates cyclin D1 in astrocytes to facilitate cell cycle progression [51]. Activation of astrocytic PAR2 can also induce ERK1/2 activation, mobilize intracellular Ca²⁺ and stimulate proliferation, although the magnitude and duration of certain PAR2 signaling effectors is different than that induced by PAR1 (Fig. 1) [52,53]. In addition, PAR1 activation can potentiate synaptic N-methyl-D-aspartic

acid (NMDA) receptor function through a mechanism that appears to involve Ca^{2+} -dependent astrocytic release of glutamate and subsequent glial–neuronal crosstalk (C.J. Lee, G. Mannaioni, H. Yuan, D.H. Woo, M.B. Gingrich, S.F. Traynelis, unpublished data). Interestingly, in astrocytes both PAR1 and PAR2 stimulate secretion of growth-regulated gene product/cytokine-induced neutrophil chemoattractant 1 (GRO/CINC-1), an important astrocytic protective factor, through different Jun N-terminal kinase (JNK) pathways [53,54].

Conclusion

Recent studies have advanced our knowledge of the function and signaling of PARs in diverse cell types including platelets, endothelial cells, neurons, astrocytes and microglia. While a role for PARs in hemostasis and thrombosis is clearly established, their functions in inflammatory and other responses associated with tissue injury have yet to be fully elucidated and deserve further research. In addition, the temporal and spatial regulation of PAR signaling is critical for a variety of cellular responses including proper cell growth and migration. Recent work has revealed new PAR activators, unexpected signaling partners and new regulatory mechanisms. The fundamental mechanisms that control protease and cell type-specific PAR signaling, however, remain to be defined.

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Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 299).

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