The Platelet PARs

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Abstract

The serine protease thrombin is a potent activator of platelets. It binds to two classes of receptors, the GPIb-IX complex and protease-activated receptors (PARs). PARs constitute a family of four G protein-coupled receptors named PARs 1–4 that mediate protease signaling in a wide variety of cells. In this chapter we describe the genomic organization and expression of PARs in platelets from humans and other species. Thrombin is the primary activator of PARs in platelets. We focus on the factors that determine the specificity and rate of cleavage PARs by thrombin, which are the initiating events of thrombin-induced platelet activation. Human platelets express PAR1 and PAR4, which have both overlapping and distinct signaling pathways. These differences have become increasingly important as therapeutics targeting PAR1 and PAR4 are developed. In addition to thrombin-PAR interactions, the activation and downstream signaling of PAR subtypes is influenced by dimerization with one another and other platelet GPCRs. We also discuss the recent identification of genetic variations that impact PAR4 signaling in humans. Finally, we highlight the differences in PAR expression on platelets across species that impact how animal models can be used as preclinical tools.

Key Points

Thrombin signaling in human platelets is mediated by protease-activated receptor 1 (PAR1) and PAR4.

PARs are G protein-coupled receptors that are activated by proteolysis of their N-terminus to expose the tethered ligand.

PAR1 and PAR4 activate overlapping and independent signaling cascades in human platelets.

PARs form homo- and hetero-oligomers that affect their activation and signaling.

Polymorphisms in the PAR4 gene (f2rl3) result in sequence variants with different reactivities, which results in altered responses to PAR4 agonists.

The expression profile of PARs on platelets varies between species, which limits the clinical translation of some animal models.

Introduction

Protease-activated receptors are members of the G protein-coupled receptor (GPCR) superfamily that are widely expressed and signal through multiple G proteins. There are four members of the PAR family of receptors, PAR1, PAR2, PAR3, and PAR4. Here, we describe the expression and activation mechanism of PAR1, PAR3, and PAR4 by thrombin and their roles in platelet signaling. PAR2 is not expressed on platelets and is not discussed in this chapter. We also give a brief overview of the expression profile of PARs in platelet from other species that are frequently used as animal models.

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Historical Perspective

In the late 1980s, several laboratories focused their work to solve the enigma of how a serine protease, thrombin, activates platelets. Thrombin is the most potent platelet agonist and plays an important role in thrombus formation and hemostasis. It is generated in the plasma by the prothrombinase complex, which catalyzes the proteolytic activation of the zymogen, prothrombin (FII), to form the active protease, thrombin (FIIa) (Krishnaswamy 2013). Binding sites with three distinct characteristics have been identified for thrombin on human platelets: high-affinity binding sites ($K_d = 0.3$ nM; 50 sites/platelet), intermediate-affinity binding sites ($K_d = 10$ nM; 1700 sites/platelet), and low-affinity nonspecific binding sites ($K_d = 3$ nM; 600,000 sites/platelet) (Harmon and Jamieson 1985). The binding sites are specific to thrombin because prothrombin does not bind to platelets. Originally, two platelet receptors were proposed as potential receptors for thrombin, glycoprotein Ib (GPIb) and glycoprotein V (GPV). However, both GPV and GPIb receptors did not fully explain how platelets response to thrombin. First, platelets from patients with Bernard-Soulier syndrome (BSS), deficient in both GPIb and GPV, respond to thrombin the same as platelets from control individuals. Second, there is no correlation between the cleavage kinetics of GPV by thrombin and the kinetics of platelet activation (Clemetson and Clemetson 1995; Kahn et al. 1999a). Finally, in the early 1990s, protease-activated receptor 1 (PAR1) was identified as the main thrombin receptor on human platelets (Vu et al. 1991a). The cloning of PAR1 lead to the identification of the three other family members (PARs 2–4).

Protease-Activated Receptor 1 (PAR1)

Protease-activated receptor 1 was the first thrombin receptor cloned in 1991 (Vu et al. 1991a). The gene encoding PAR1 (*f2r*) is located in chromosome 5 (5q13). PAR1 has a genomic structure that contains two exons separated by an intron of approximately 15 kb (Kahn et al. 1998a). The *f2r* gene encodes for a 425 amino acid protein with seven transmembrane domains. The general activation mechanism common to all members of the PAR family were largely worked out for PAR1 (Fig. 1). The N-terminal domain of PAR1 is oriented to the extracellular space and contains the recognition site(s) for thrombin. Following cleavage by thrombin at arginine 41 (. . .$LDPR_{41}$/SFLLRN. . .), the new N-terminal exodomain (SFLLRNP. . .) acts as tethered ligand that binds to the second extracellular loop of PAR1. This leads to a conformational change in the receptor, which initiates signal transduction (Nanevicz et al. 1995). The synthetic peptide,

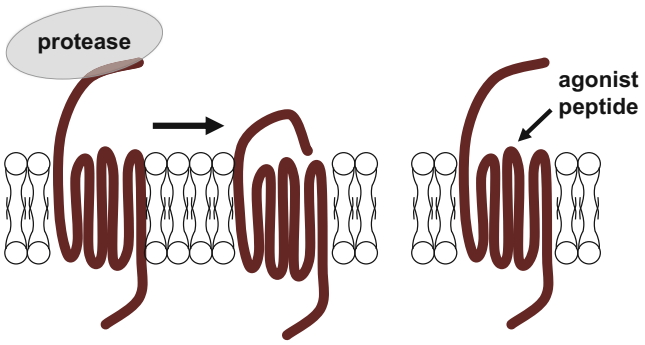

Fig. 1 A schematic of PAR activation by the tethered ligand. The protease binds and cleaves the N-terminal exodomain. The newly formed N-terminus binds to the extracellular loops to initiate downstream signaling events (*left panel*). Experimentally, PARs can be activated via agonist peptides that mimic the cleaved N-terminus (*right panel*). These are often referred to as TRAPs (thrombin receptor-activating peptides)

SFLLRN, mimics the first six amino acids of newly formed N-terminus and can activate PAR1 independent of cleavage (Gerszten et al. 1994). The PAR activation peptides, sometimes referred to as thrombin receptor-activating peptides (TRAPs), have been used experimentally to selectively activate PARs on cells to study the signaling events that are downstream to specific PAR family members. One caveat is that SFLLRN will also activate PAR2. The peptide TFLLRN should be used for cells co-expressing PAR1 and PAR2, such as endothelial cells.

The simplistic view of PAR activation by the tethered ligand is that the N-terminus bends over and activates the receptor by binding extracellular loop 2. However, NMR and mutagenesis studies with the PAR1 exodomain suggest that the N-terminus actually folds back on itself to form a secondary structure that is required for activation (Seeley et al. 2003). It is not known if this is a general mechanism of PAR activation as analogous studies have not been reported for other PAR family members. Although PAR1 was originally identified as the thrombin receptor, it is widely expressed and can be cleaved by multiple proteases to activate distinct signaling pathways through biased agonism (see discussion below) (Koukos et al. 2011; Zhao et al. 2014). It is not known how the alternative cleavage sites influence the secondary structure of the PAR1 N-terminus.

The unique activation mechanism of PARs is irreversible, which prohibits receptor recycling following activation. Following stimulation, PAR1 undergoes desensitization due to receptor internalization (Molino et al. 1997; Brass et al. 1992). A key regulatory step of PAR1 signaling is phosphorylation of the C-terminal tail (Shapiro et al. 2000). More recent studies have also linked PAR1 internalization to ubiquitination and glycosylation (Chen et al. 2011; Soto and Trejo 2010; Russo et al. 2009a; Wolfe et al. 2007).

Protease-Activated Receptor 3 (PAR3)

Protease-activated receptor 3 was cloned in 1997 as the second thrombin receptor and third member of the PAR family (Ishihara et al. 1997). The gene encoding PAR3, *f2rl2*, co-localizes with *f2r* (the PAR1 gene) at chromosome 5q13. It is located at 5′ of *f2r* and separated by less than 25 kb (Schmidt et al. 1996; Kahn et al. 1998a). The common locus of the genes for PAR1 (*f2r*), PAR2 (*f2rl1*), and PAR3 (*f2rl2*) suggests a common ancestral gene for this family of receptors. The genomic organization of *f2rl2* is similar to *f2r*, with one small exon and one large exon. In contrast to *f2r*, the two exons of *f2rl2* are separated by a small intron of approximately 4.5 kb (Kahn et al. 1998a).

The PAR3 mRNA encodes for a 373 amino acid protein, which shares 27 % amino acid sequence identity with PAR1. Similar to PAR1, the N-terminal exodomain of PAR3 has two thrombin recognition sites; the cleavage site is at lysine 38 (. . .LPIK$_{38}$/TFRGAP. . .) and a hirudin-like sequence (FEEFP) that binds thrombin's exosite I (Ishihara et al. 1997; Ayala et al. 2001; Bah et al. 2007). A major difference between PAR1 and PAR3 is that the C-terminal tail of PAR3 is significantly shorter than that of PAR1 (13 versus 51 amino acids). The shorter C-terminal tail likely affects the coupling of PAR3 to intracellular signaling machinery and subsequent signal transduction for platelet activation. PAR3-activating peptide, TFRGAP, does not activate human platelets, *Xenopus* oocytes, or COS7 cells transfected with human PAR3, suggesting that it does not have a signaling function on its own (Andersen et al. 1999; Ishihara et al. 1997). In human platelets, the expression level of PAR3 is substantially lower than PAR1, 150–200 copies versus 1500–2000 copies on the surface, respectively (Brass et al. 1992; Schmidt et al. 1998). It has been difficult to define a functional role for PAR3 on human platelets (Ishihara et al. 1997). In contrast, PAR3 is highly expressed on mouse platelets in which it regulates the sensitivity and response of PAR4 at both low and high thrombin concentrations (Nakanishi-Matsui et al. 2000; Arachiche et al. 2013a). Similar to human platelets, a direct signaling role for PAR3 has not been described.

Protease-Activated Receptor 4 (PAR4)

Protease-activated receptor 4 (PAR4) was the fourth member of PAR family and the third thrombin receptor identified (Xu et al. 1998; Kahn et al. 1998b). In contrast to *f2r* and *f2rl2*, the gene encoding PAR4, *f2rl3*, maps to chromosome 19p12. The *f2rl3* gene is also organized into two exons, but the intron separating the two exons is small (~0.25 kb) compared to other PARs (Kahn et al. 1998a). The expression of PAR4 mRNA was detected in platelets and a number of other human tissues such as the lung, pancreas, thyroid, testis, and small intestine. PAR4 expression was not detected in the brain, kidney, spinal cord, and peripheral blood leukocytes (Kahn et al. 1999b; Xu et al. 1998).

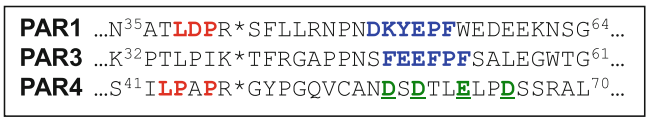

Fig. 2 Schematic diagram of PAR1, PAR3, and PAR4 exodomains. PAR1 and PAR3 primary sequence with amino acids that interact with the thrombin cleavage site highlighted in *red* and the hirudin-like sequence highlighted in *blue*. PAR4 primary sequence with amino acids that interact with the thrombin cleavage site highlighted in *red* and the anionic cluster highlighted in *green* and *underlined*

PAR4 is a protein that is 385 amino acids in length with 27 % and 30 % amino acid sequence identity with PAR1 and PAR3, respectively (Xu et al. 1998). Thrombin activates PAR4 by the cleavage of the N-terminal of PAR4 at arginine 47 (. . .LPAPR$_{47}$/GYPGQV. . .) (Fig. 2). The synthetic peptide (GYPGQV) corresponding to the unmasked amino terminus of PAR4 also activates the receptor. However, peptide library screens demonstrated that the peptide AYPGKF is more potent and is commonly used experimentally (Hollenberg and Saifeddine 2001).

PAR1 has been more extensively studied than PAR4, which has lead to the development of the FDA-approved drug vorapaxar (Baker et al. 2014). However, recently it has been recognized that PAR4 has unique signaling properties that may make PAR4 an attractive target for therapeutics (Kuliopulos and Covic 2003; Young et al. 2013; Mumaw et al. 2014). Further, the identification of PAR4 sequence variants that result in receptors with different reactivities highlights the importance that each receptor contributes to thrombin signaling. Finally, since PAR1 is not expressed on mouse platelets, signaling ascribed to PAR4 in mouse studies need to be interpreted with caution. For example, the C-terminus of mouse PAR4 has properties similar to human PAR1, which affects binding of some antagonists (Aisiku et al. 2015). These differences are discussed below.

Activation of PARs by Thrombin

Much effort has been made to characterize the molecular mechanisms by which thrombin interacts with its substrates. In general, thrombin's interaction with its substrates consists of three parts: (1) it binds via its anionic binding exosite I to a site on some of its substrates termed a "hirudin-like sequence," (2) it binds through amino acids that surround the active site to some amino acids at the P5-P2 positions on the substrate, and (3) thrombin's active site interacts

with the substrate P1-P1′ position. The rate of cleavage of PARs by the activating protease is the rate-limiting step for signaling.

In addition to binding thrombin's active site, PAR1 has a hirudin-like sequence ($D^{50}KYPEK^{55}$) that binds thrombin's exosite I which induces allosteric effects on thrombin, lowering the energy required for PAR1 cleavage (Fig. 2) (Liu et al. 1991; Ayala et al. 2001; Jacques et al. 2000). The importance of the hirudin-like sequence has been confirmed with thrombin exosite mutations as well as PAR1 exodomain mutations (Ayala et al. 2001; Jacques et al. 2000; Vu et al. 1991b; Myles et al. 2001). Further, mutations around the thrombin cleavage site do not dramatically affect the K_m of thrombin cleaving PAR1 due to the exosite I binding region. However, the k_{cat} is reduced sevenfold when Leu^{38} at P4 is mutated to alanine and twofold when Pro^{40} at P2 is mutated (Fig. 2) (Nieman and Schmaier 2007). When PAR1 peptides were co-crystallized with thrombin, structures were solved in which the active site of thrombin interacted with $L^{38}DPR^{41}$ or the exosite I region interacted with $K^{51}YEPF^{55}$. However, none of these structures had the exosite I and the active site simultaneously filled (Mathews et al. 1994). The hirudin-like sequence ($K^{51}YEPF^{55}$) induces a change of conformation when the Ala^{190}-Gly^{197} region of thrombin with the Glu^{192} side chain becomes disordered, helping to accommodate the negatively charged Asp at P3 for PAR1. The crystals in which the active site is filled by the $L^{38}DPR^{41}$ sequence shows that Leu^{38} occupies the aryl-binding pocket formed by Ile^{174} and Trp^{215} as predicted by Bode et al. (1992). The intervening sequence between $L^{38}DPR^{41}$ and $K^{51}YEPF^{55}$ of PAR1 ($F^{43}LLRNP^{48}$) is disordered with no electron density (Mathews et al. 1994). Using the data from the two sets of crystals in Mathews et al. (PDB ID codes 1NRS and 1NRN), Huntington has proposed models for a single PAR1 peptide interacting with thrombin's active site and exosite I simultaneously, which has largely been confirmed by more recent structural studies (Huntington 2005; Gandhi et al. 2010).

PAR4 does not have a hirudin-like sequence (Fig. 2) (Xu et al. 1998). Based on studies with peptides and recombinant exodomains, the primary sites of PAR4 interaction with α-thrombin is at the thrombin cleavage site. In particular, amino acids Leu^{43} at P5, Pro^{44} at P4, and Pro^{46} at P2 are important α-thrombin interaction sites (Cleary et al. 2002; Jacques and Kuliopulos 2003; Nieman and Schmaier 2007). However, individual point mutations at Leu^{43}, Pro^{44}, or Pro^{46} do not influence thrombin binding (i.e., did not influence the K_m) but did reduce the rate of cleavage indicating that Leu^{43}, Pro^{44}, or Pro^{46} are important for orienting PAR4 in the active site of thrombin for efficient cleavage (Nieman and Schmaier 2007). More importantly, these data also suggest that, like PAR1, PAR4 has extended contacts with α-thrombin that minimize the influence of the individual amino acids at the cleavage site (Fig. 1) (Nieman and Schmaier 2007). Earlier work by Jacques and Kuliopulos, using purified exodomains, demonstrates that mutations of the anionic cluster in the PAR4 exodomain (Asp^{57}, Asp^{59}, Glu^{62}, Asp^{65}) (see Fig. 2) decreased the K_m of thrombin binding fourfold from 56 to 208 nM (Jacques and Kuliopulos 2003). Further experiments demonstrate that the anionic cluster stabilizes the interaction with thrombin by slowing the dissociation rate (Jacques and Kuliopulos 2003). These functional data are supported by structural and modeling studies. Ayala et al. showed via molecular modeling using peptides that Leu^{43} may be important for interaction with thrombin residues Leu^{99}, Ile^{174}, and Trp^{215} (Ayala et al. 2001). However, NMR studies with PAR4 peptides by Cleary et al. demonstrate that this leucine is flexible and can interact with thrombin as well as Pro^{44} or Pro^{46} to stabilize secondary structure at the thrombin cleavage site of PAR4 (Cleary et al. 2002). Crystallography studies show that PAR4 interacts with thrombin's gamma (autolysis) loop (Bah et al. 2007).

PAR4 is not an efficient thrombin substrate when expressed on cells alone and requires approximately tenfold more thrombin for activation (Jacques and Kuliopulos 2003; Nieman 2008). However, when PAR4 is co-expressed with PAR1 in heterologous systems as it is on human platelets, the rate of PAR4 cleavage is enhanced six- to tenfold (Jacques and Kuliopulos 2003; Nieman 2008; Arachiche et al. 2013b). The enhanced rate of PAR4 cleavage is similar that first described for the functional interaction between PAR3 and PAR4 on mouse platelets (Nakanishi-Matsui et al. 2000). In this model, thrombin remains bound to the hirudin-like sequence following proteolysis at the cleavage site, which enhances the activation of an adjacent PAR4 (Fig. 3). There are two potential mechanisms for this model. First, the higher-affinity binding site on PAR1 or PAR3 (the hirudin-like sequence) may increase the local concentration of thrombin near the platelet surface to facilitate the activation of PAR4. Alternatively, based on biochemical and structural data with other tight exosite I binders, PAR1's hirudin-like sequence may induce thrombin into the protease conformation with an open active site to facilitate PAR4 cleavage (Huntington 2012; Kamath et al. 2010). The latter model is also supported by structural models of murine thrombin bound to peptides from PAR3 and PAR4 (Bah et al. 2007).

Thrombin Signaling in Human Platelets

The responses of platelets to thrombin stimulation are many and varied. PAR1 and PAR4 cooperate to mediate the full range of thrombin signaling in human platelets by coupling to multiple heterotrimeric G proteins (Kahn et al. 1999b).

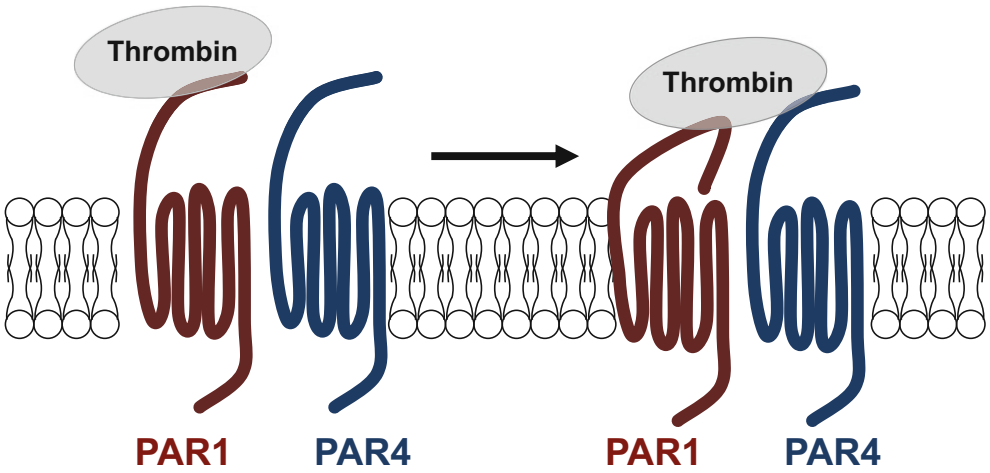

Fig. 3 PAR1 serves as a cofactor for PAR4 activation by thrombin on human platelets. The rate of PAR4 cleavage is enhanced by co-expression of PAR1 (human platelets) or PAR3 (mouse platelets) via the hirudin-like sequence; see text for details

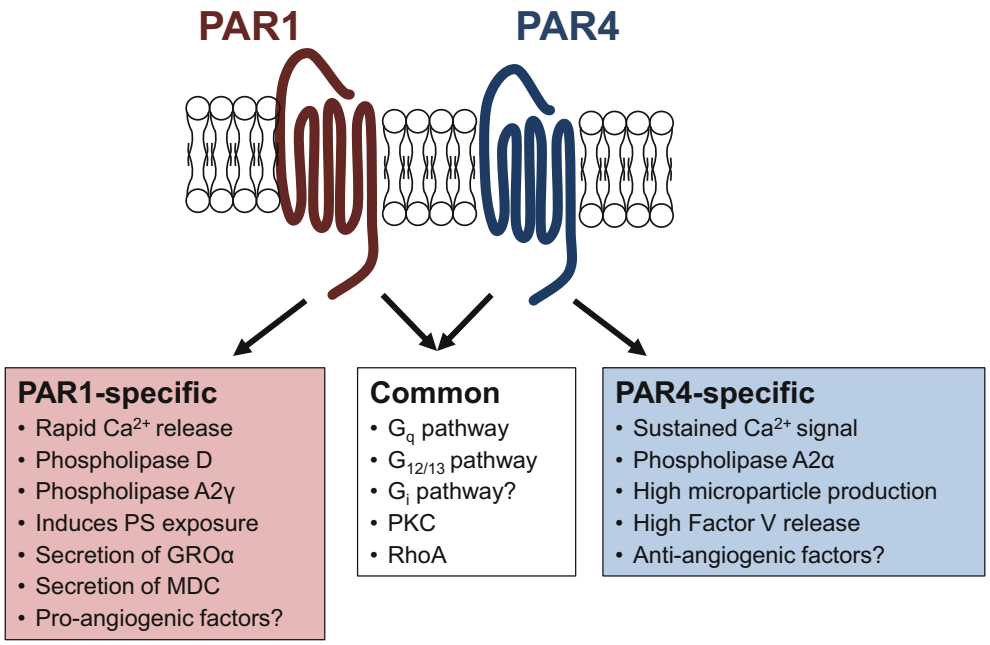

Fig. 4 PAR1 and PAR4 have overlapping and distinct downstream signaling events. PAR1 and PAR4 both signal through multiple G proteins; however, the duration and kinetics of signaling can differ

PAR1 and PAR4 have several overlapping signaling functions, which led to the original hypothesis that PAR4 is a redundant, backup receptor. Several studies have since shown these receptors can activate unique signaling pathways and, in some cases, the same pathways with distinct kinetics (Fig. 4).

Overlapping Signaling Between PAR1 and PAR4

In human platelets, PAR1 and PAR4 both transduce signals through $G\alpha_q$ and $G\alpha_{12/13}$. The activation of $G\alpha_q$ stimulates the formation of inositol triphosphate (IP3) and diacylglycerol (DAG), which induces intracellular calcium mobilization and protein kinase C (PKC) activation, respectively (Hung et al. 1992; Offermanns et al. 1997). This pathway controls a variety of platelet responses including granule secretion, integrin activation, and platelet aggregation in platelets. The activation of $G\alpha_{12/13}$ mediates Rho guanine nucleotide exchange factors and RhoA signaling pathways, which controls platelet shape change (Moers et al. 2003; Huang et al. 2007). $G\alpha_i$ signaling in platelets mediates inhibition of adenylate cyclase activity but also induces platelet shape change, secretion, and calcium mobilization. The direct interaction between PARs and G_i in human platelets is controversial. PAR1 directly couples to G_i in COS7 cells transfected with PAR1 (McCoy et al. 2012) and, in some cases, platelets (Voss et al. 2007). In other studies, PAR1 and PAR4 did not couple directly to G_i. Here, the G_i pathway was mediated by secondary release of ADP, which acts on the G_i-coupled ADP receptor, P2Y12 (Jantzen et al. 2001; Kim et al. 2002, 2006).

Calcium Mobilization

The most dramatic differences between PAR1 and PAR4 signaling in platelets is the kinetics and duration of intracellular Ca^{2+} signaling (Covic et al. 2000; Vaidyula and Rao 2003). Prior to the identification of PAR4, it was recognized that Ca^{2+} mobilization in thrombin-stimulated platelets was different from that of PAR1 agonist peptide-stimulated platelets. Further studies revealed that the PAR1 agonist peptide is a partial agonist of human platelets (Lau et al. 1994). The initial increase in intracellular Ca^{2+} was the same for thrombin and the agonist peptide. In contrast, when platelet was stimulated with the PAR1 agonist peptide in the absence of extracellular Ca^{2+}, the Ca^{2+} flux returned to baseline levels faster and induced a reduced level of lysosome release. Finally, the overall magnitude and duration of the Ca^{2+} signal was greater in thrombin-stimulated platelets (Heemskerk et al. 1997). The identification of PAR4 reconciled these differences when it was demonstrated that there is a distinct wave of Ca^{2+} signaling from PAR4 (Covic et al. 2000). The prolonged Ca^{2+} stimulus associated appears to be required for stable clot formation and full spreading on fibrinogen in response to thrombin in a p38- and ERK1/2-dependent manner (Covic et al. 2002; Mazharian et al. 2007). The proposed mechanism for sustained signaling from PAR4 is that PAR4 is internalized more slowly compared to PAR1. In cultured fibroblast, 50 % of PAR1 is internalized following stimulation compared to 20 % of PAR4 (Shapiro et al. 2000).

Cooperation Between Thrombin and ADP Signaling

PAR1 and PAR4 also synergize with ADP signaling in platelets. One study has shown that PAR4, but not PAR1 signaling to Akt, is dependent on P2Y12, particularly under conditions of limited calcium concentration (Holinstat et al. 2006). A number of other reports have found results suggesting that PAR1 synergizes independently with P2Y12, rather than PAR4 (Resendiz et al. 2007; Wu et al. 2010; Jiang et al. 2013). PAR1 but not PAR4 directly influenced ADP-induced platelet granule secretion and second wave of aggregation. Blocking PAR1 activation by SCH79797 abolished the ATP secretion, $\alpha_{IIb}\beta_3$ activation, P-selectin expression, and the second wave of platelet aggregation associated with partial disaggregation. In contrast, PAR4 antagonist tcY-NH2 had no effect on these responses (Jiang et al. 2013). Furthermore, selective activation of PAR1 by SFLLRN together with collagen enhanced the increase in exposure of procoagulant phosphatidylserine (PS) exposure on the surface of platelets, as simultaneous stimulation of platelets with thrombin and collagen. The selective activation of PAR4 by the agonist peptide, GYPGQV, resulted in less PS exposure on the platelet surface (Andersen et al. 1999). In addition, stimulating platelets with ADP prior to SFLLRN produced a much greater increase in subpopulation of platelets that are PS positive compared to simultaneous stimulation of PAR1/P2Y12 (Shakhidzhanov et al. 2015). In sum, these studies indicate that P2Y12 and PAR1 signaling work in cooperation. Finally, in addition to the interaction of downstream signaling pathways, P2Y12 and PAR4 have a direct physical interaction that influences arrestin recruitment (see below) (Khan et al. 2014; Li et al. 2011).

Membrane Lipids and PAR1–PAR4 Downstream Signaling

PAR1 and PAR4 differentially regulate membrane lipid signaling. The role of sphingolipids has been demonstrated in both platelet function and platelet production (Shrimpton et al. 2002; Zhang et al. 2012b). Sphingomyelin (SM) is one of the major sphingolipids present in the plasma membrane and is hydrolyzed by sphingomyelinase (SMase) enzyme into ceramide and phosphorylcholine. Two types of SMase have been identified in platelets, the lysosomal phosphodiesterase acid SMase (A-SMase) and the membrane-bound neutral SMase (N-SMase); both enzymes regulate mouse and human platelets (Munzer et al. 2014; Chen et al. 2013). Thrombin or PAR4 agonist peptide, but not PAR1 agonist peptide, results in increased association of N-SMase with PAR4 in human platelets. The activation of N-SMase induced the generation of ceramide, which acts as a second messenger to induce the activation of the p38-MAPK-NF-kB signaling pathway in platelets (Chen et al. 2013). Thus far, the role of PAR1 or PAR4 in regulating acid sphingomyelinase (A-SMase) has not been demonstrated.

Arachidonic acid (AA) is another lipid membrane, which plays an important role in platelet function. AA is liberated form glycerophospholipid (GPL) via the action of phospholipase A2 (PLA2) and transformed to thromboxane A2 (TXA2) by sequential action of cyclooxygenase-1 (COX-1) and TXA2 synthase. Thrombin stimulates the generation of thromboxane A2 (TXA2), which, in turn, activates platelets via the thromboxane receptor (TP) and amplifies platelet activation to cause irreversible aggregation. PAR4 stimulation results in significantly greater TXA2 generation compared to PAR1 (Holinstat et al. 2011). Platelets express several PLA_2 isoform, including Ca^{2+}-sensitive 85 kDa cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$), Ca^{2+}-insensitive 14 kDa secretory PLA_2 ($sPLA_2$), and Ca^{2+}-independent PLA2 ($iPLA_2\gamma$). It has been shown that thrombin-induced AA production in human platelets is dependent on $cPLA_2\alpha$ but not $sPLA_2$ (Bartoli et al. 1994).

The platelets from cPLA2α-deficient mice or patient with inherited deficiency of cPLA2α present a decrease in eicosanoid biosynthesis, such as prostaglandins, thromboxanes, and leukotrienes, which alters platelet function (Adler et al. 2008). In addition, mice lacking iPLA2γ present with a prolonged bleeding time and are protected from pulmonary thromboembolism (Yoda et al. 2014). In very recent work, a specific inhibitor of cPLA2α, giripladib, selectively inhibited PAR4 but not PAR1-mediated P-selectin expression in human platelets. However, specific inhibition of iPLA2γ with bromoenol lactone (BEL) significantly reduced P-selectin expression after PAR1, but not PAR4 activation in human platelets (Duvernay et al. 2015). Studies with human platelets show that inhibition of the phosphatidylcholine (PC)-derived phosphatidic acid (PA) formation by phospholipase D (PLD) inhibits platelet activation by PAR1-activating peptide. Thrombin or PAR4-activating peptides are insensitive to this inhibition. Furthermore, PAR1 but not PAR4 signals through phosphoinositide 3-kinase (PI3K) to activate integrin $\alpha_{IIb}\beta_3$ and induce platelet aggregation (Holinstat et al. 2007; Voss et al. 2007).

Granule Secretion and PAR1–PAR4 Downstream Signaling

Human platelets contain three types of storage granules, α-granules, dense granules, and lysosomes. The presence of distinct of α-granules with either pro-angiogenic or anti-angiogenic factors is controversial with studies supporting both selective and random secretion of granule contents. The differential release of pro-angiogenic and anti-angiogenic factors from platelets stimulated with PAR1 versus PAR4, respectively, was first described by Ma and colleagues (2005). The PAR4 agonist peptide stimulated the release of endostatin but suppressed the release of VEGF. In contrast, the PAR1 agonist peptide stimulated the release of VEGF and suppressed the release of endostatin. Curiously, stimulation with thrombin did not release either factor. Other studies also support a differential release of anti-angiogenic versus pro-angiogenic factors that are not due solely to either differential signal strength or kinetics of the respective agonists (Italiano et al. 2008; Chatterjee et al. 2011). Notably, PAR4-induced secretion of SDF-1 and endostatin were PI3K and Akt dependent, while PAR1-induced SDF-1 secretion was not (Chatterjee et al. 2011). PAR1 and PAR4 can also influence the degree of secretion. For example, PAR4 stimulation with the agonist peptide, AYPGKF, enhanced the surface expression of Factor V (1.6-fold) and P-selectin (0.8-fold) compared to PAR1 activation with SFLLRN (Duvernay et al. 2013). PAR4 activation also induced a threefold greater production of platelet microparticles compared with PAR1 activation. The RhoA pathway inhibitor Y-27632 reduced Factor V translocation and microparticle release downstream PAR4 stimulation to levels observed with PAR1 stimulation. These data indicate that PAR4 is mediating these events through the $G_{12/13}$-RhoA signaling axis. PAR4 activation also releases more CD40L from α-granules compared to PAR1. Conversely, PAR1 activation releases more growth-regulating oncogene-α (GRO-α) and macrophage-derived chemokine (MDC), compared to PAR4 (Nguyen et al. 2015).

In contrast, several studies have demonstrated random distribution of proteins within granules and that PAR1 and PAR4 stimulation leads to random release of granule contents from human platelets. A detailed analysis of α-granule proteins with quantitative immunofluorescence co-localization with pair-wise comparisons demonstrates the presence of one type of α-granule with random packing (Kamykowski et al. 2011). An analysis of the rates of secretion of several granules shows that the differences in α-granule release observed between PAR1 and PAR4 are based on the kinetics of granule release due to the strength of the agonist (Jonnalagadda et al. 2012). Furthermore, the RhoA activation downstream $G_{12/13}$ and G_q induced the same level of dense granule release in response to PAR1 or PAR4 activation (Jin et al. 2009). Recent work from van Holten et al. using mass spectrometry (MS)-based quantitative proteomic analysis and enzyme-linked immunosorbent assay (ELISA) shows that PAR1 or PAR4 activation of platelets results in the same α-granule release (van Holten et al. 2014). Finally, it is difficult to conclude if PAR1 and PAR4 induced similar or different protein mobilization, because the controversial results obtained from these studies might be due to the various techniques used to analyze platelet release, differences in agonist concentration, or difference in the number of human platelet samples analyzed.

Biased Signaling of PARs

GPCRs can signal through G protein and arrestin pathways. Biased agonists are those that preferentially activate specific pathways downstream of the receptor (Urban et al. 2007). In contrast, neutral agonists do not discriminate which pathways are activated. Biased signaling downstream of PARs can be mediated by cofactors, alternative cleavage sites, or pharmacologically (Zhao et al. 2014; Lin et al. 2013; Aisiku et al. 2015; Dowal et al. 2011). Studies aimed at understanding and directing the multitude of signaling events have largely been focused in endothelial cells. In this review we will focus on biased signaling as it relates to platelets.

Biased Signaling from PARs in Platelets

Thrombin is a neutral agonist for PAR1 and does not discriminate the downstream pathways that are activated. The first description of a PAR1 ligand that demonstrated specific signaling events in platelets was the activation peptide YFLLRNP (Rasmussen et al. 1993; Bauer et al. 1999). At low concentrations, this version of the PAR1 activation peptide induces shape change through $G_{12/13}$ but does not mediate G_q signaling and, as a result, does not induce full aggregation. Observations such as these opened the possibility of targeting specific pathways downstream of PARs. Since PAR1 activation has many, and sometimes divergent, cellular responses, inhibiting all downstream signaling with an orthosteric inhibitor such as vorapaxar (Zontivity) may not be ideal. An alternative approach that has be pursued by Flaumenhaft and colleagues is to screen for allosteric modulators of PAR1 that spare the cytoprotective signaling of PAR1 while blocking pathways that are detrimental to cell survival (Aisiku et al. 2015; Dowal et al. 2011). Their primary focus has been on PAR1 signaling in endothelial cell; however, the parmodulin compounds also selectively block G_q signaling in platelets. This family of compounds binds to the eighth helix on the cytoplasmic face of the receptor where they alter the interactions between the receptor and the G-alpha subunits.

The traditional role of arrestins is to regulate GPCR trafficking. The reports of arrestin function in platelets have been less straightforward. The internalization of P2Y receptors in an arrestin-dependent manner results in shutting down signaling (Nisar et al. 2012, 2011). In contrast, other reports suggest a direct signaling function of arrestin that promotes platelet activation (Schaff et al. 2012; Li et al. 2011). The specific contributions of arrestins to PAR signaling have been limited to PAR4 (Khan et al. 2014; Li et al. 2011). Recruitment of arrestin-2 to PAR4 is mediated by PAR4-P2Y12 heterodimerization where it has a positive signaling role. In mice that have arrestin-2 deleted, the platelet function is enhanced, and the time to thrombosis is shortened due to increased Src family kinase activation. These data support a direct signaling role for arrestin-2 rather than the expected desensitization of GPCR-mediated signaling that is expected.

Activation PARs by Proteases Other Than Thrombin

In addition to thrombin, several other serine proteases are capable of activating PARs such as factor Xa, plasmin, matrix metalloproteinases 1 and 13, elastase, activated protein C (APC), proteinase-3, granzyme, cathepsin G, and calpain (Zhao et al. 2014). Depending the cleavage site, PARs can be activated or inactivated by these proteases. Further, the alterative cleavage sites can generate novel tethered ligands that initiate specific signaling pathways. The panel of proteases activating PARs has been described for a variety of cell types. Here, we will focus on protease cleavage of PAR1 and PAR4 in the context of platelet function. The reader is directed to recent comprehensive reviews for other contexts (Russo et al. 2009b; Hollenberg et al. 2014; Zhao et al. 2014).

Factor Xa is directly upstream of thrombin in the coagulation cascade and can directly activate PARs (Camerer et al. 2002, 2000; Ruf et al. 2003). Early studies by Sinha et al. show that the pretreatment of platelet-rich plasma with FXa inhibited thrombin-induced platelet aggregation and TXA2 generation (Sinha et al. 1983). One potential caveat is distinguishing between FXa activation of prothrombin (which can subsequently activate PAR1) from direct activation of PAR1 by FXa. However, if the thrombin concentration is increased tenfold over FXa or if FVa is blocked on the surface of platelets using a specific anti-FVa antibody, the inhibition of TXA2 synthesis and platelet aggregation induced by FXa is reversed.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are secreted as zymogens and, upon activation, degrade extracellular matrix proteins during tissue repair and cancer invasion (Woessner 1999). Matrix metalloproteinase-1 (MMP-1) directly activates platelets via PAR1. Stimulation of platelets with collagen results in the conversion of the inactive proMMP-1 to active MMP-1. Once activated, MMP-1 is capable of inducing platelet signaling by direct cleavage of PAR1 at a noncanonical site, Asp^{39} ($TLD_{39}PR_{41}SFLLRN$), that is distinct from canonical cleavage site of thrombin at Arg^{41} (Trivedi et al. 2009; Austin et al. 2013). In human platelets, the cleavage of PAR1 at Asp^{39} by MMP-1 induces $G_{12/13}$-Rho, p38 MAPK pathways, and shape change. However, intracellular calcium mobilization and platelet aggregation in response to MMP-1 are less potent than with thrombin (Austin et al. 2013; Trivedi et al. 2009). It should be noted that other cleavage sites on PAR1 have been reported for MMP-1 (Boire et al. 2005; Nesi and Fragai 2007).

The fibrinolytic enzyme plasmin cleaves both PAR1 and PAR4 on platelets. Four cleavage sites have been identified on the PAR1 exodomain (Arg^{41}, Arg^{70}, Lys^{76}, and Lys^{82}) (Kuliopulos et al. 1999). Kuliopulos and colleagues showed that although plasmin is capable of cleaving PAR1 at the canonical thrombin site, the predominant result is inactivation of PAR1 by truncating the tethered ligand at the distal sites. In the presence of a PAR1 inhibitor, plasmin stimulates platelet aggregation and shape change in human platelets via PAR4. Plasmin cleaves PAR4 at canonical thrombin cleavage site Arg^{47} (. . .$LPAPR_{47}$GYPGQV. . .) to generate PAR4 tethered ligand peptide (GYPGQV) (Quinton et al. 2004).

Cathepsin G is a serine protease found in the dense granules of neutrophils and is secreted upon neutrophil activation that cleaves both PAR1 and PAR4. The analysis of the N-terminal exodomain of PAR1 identified three potential cleavage site for cathepsin G: ($TLDPR_{41}SF_{43}LLRN...F_{55}..$). Preincubation of platelets with cathepsin G completely abolish thrombin-induced calcium mobilization (Parry et al. 1996). This is due to cathepsin G cleavage at Phe^{55} which leads to a loss of the PAR1 tethered ligand. Cathepsin G also cleaves PAR4 and induces calcium mobilization in human platelets and in PAR4 transfected fibroblasts (Sambrano et al. 2000). The story in mouse platelets is different. Cathepsin G blocks signaling by low thrombin concentrations by cleaving PAR3 and preventing it from acting as a cofactor for PAR4 (Cumashi et al. 2001). Cumashi and colleagues also showed that cathepsin G does not activate mouse PAR4.

Physical Interactions Between PARs

The molecular organization of GPCRs within the plasma membrane is controversial (Vischer et al. 2015). Homo- and heterodimers of GPCRs may influence signaling and can be thought of as allosteric modulators (Milligan and Smith 2007). The best evidence for a functional interaction between PAR homodimers on platelets is the dominant negative effect of PAR4 sequence variants that have low reactivity, which corroborates molecular studies (Edelstein et al. 2014; de la Fuente et al. 2012). Physiologically, there are examples of PAR heterodimers influencing the rate of activation, downstream signaling, and trafficking of the receptors in a diverse set of environments including platelets, smooth muscle cells, endothelial cells, and podocytes (Fig. 5) (Lin et al. 2013). As discussed above, the coordinated activity between PARs was first described for PAR3 and PAR4 on mouse platelets where PAR3 serves as a cofactor to enhance the rate of PAR4 cleavage by thrombin by approximately tenfold (Nakanishi-Matsui et al. 2000). An analogous mechanism was later demonstrated for PAR1 and PAR4 on human platelets (Leger et al. 2006; Nieman 2008). Each of these cases are dependent on PAR4 heterodimerization with PAR1 (human) or PAR3 (mouse) (Leger et al. 2006; Arachiche et al. 2013a, b).

PAR4, but not PAR1, also forms agonist-dependent heterodimers with another platelet GPCR, the ADP receptor P2Y12 (Li et al. 2011; Khan et al. 2014). The interaction between P2Y12 and PAR4 enhances the recruitment of β-arrestin-2 to PAR4 and mediates sustained signaling through Akt in human platelets. The PAR4-P2Y12 dimerization appears to be important to stabilize platelet plug formation. The interaction interface of the PAR4-P2Y12 heterodimer has been mapped to transmembrane helix 4 of PAR4. A mutation in PAR4 at the heterodimer interface disrupted the interaction with P2Y12 and prevents β-arrestin-2 recruitment and Akt activation (Khan et al. 2014). These data indicate that P2Y12 and PAR1 are competing with each other to interact with PAR4 as both receptors share the same heterodimer interface with PAR4 as well as the PAR4 homodimer interface (de la Fuente et al. 2012; Arachiche et al. 2013b; Khan et al. 2014). These interactions have the potential to not only influence platelet signaling but also how patients respond to therapies (Fig. 5) (Mumaw and Nieman 2014).

In addition to enhancing PAR4 activation, PAR3 can influence PAR4 signaling. PAR3-deficient mice have a 1.6-fold increase in the maximum Ca^{2+} mobilization and an increase in PKC activation but no effect on RhoA-GTP activation compared to platelets from wild-type mice. These results demonstrate that PAR3 regulates PAR4/G_q signaling pathway via a direct interaction with PAR4 indicating that dimerization of PARs may regulate coupling of G proteins to the receptor to influence downstream signaling. In addition to platelets, PAR3 also regulates PAR1 signaling in endothelial cells (McLaughlin et al. 2007; Stavenuiter and Mosnier 2014; Burnier and Mosnier 2013).

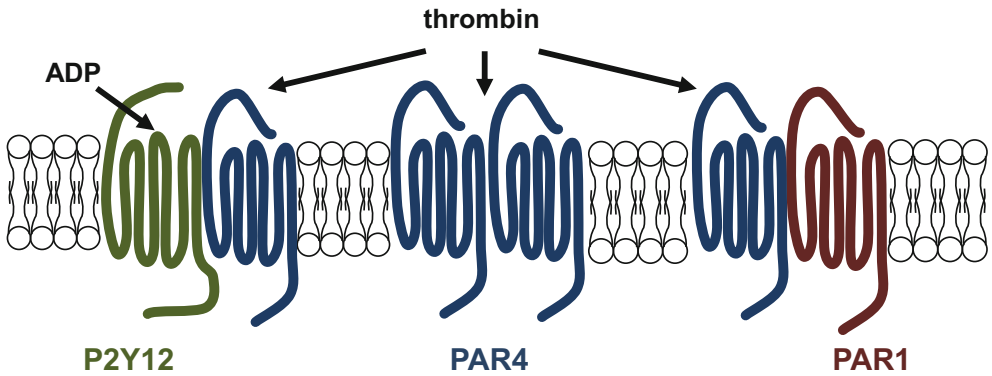

Fig. 5 The physical interaction between PARs. PARs for homo- and hetero-oligomers between PAR family members and other platelet GPCRs. These interactions influence both the rate of activation and signaling. Oligomerization also has the potential to influence the response to therapies. Note: PAR1 and P2Y12 also form homo-oligomers but are not shown in the figure for clarity

Polymorphisms and Sequence Variants

Single-nucleotide polymorphisms have been described for both PAR1 and PAR4. The challenge with SNPs is establishing a direct link from the identified polymorphisms to receptor expression or function and ultimately to a physiological output. One of the first described was a PAR1 polymorphism in an intron that affects PAR1 density on platelets and decreased platelet response to PAR1 agonists in individuals (Dupont et al. 2003). However, in a recent clinical study with 660 patients who underwent percutaneous coronary intervention (PCI), there was no evidence of increased major adverse cardiovascular events (MACE) or bleeding risk correlated with the polymorphism (Friedman et al. 2015).

The heritable interindividual variation in platelet reactivity has been directly linked to PAR4 (Bray et al. 2007; Edelstein et al. 2013, 2014; Tourdot et al. 2014). The Platelet RNA And eXpression 1 (PRAX1) study was designed to examine mRNAs and microRNAs associated with this difference in 154 healthy individuals who self-identify as black or white. In this population, Edelstein et al. showed that the black individuals had increased platelet response to PAR4 stimulation, higher expression of phosphatidylcholine transfer protein (PC-TP), and lower levels of miR-376c (Edelstein et al. 2013). The opposite was observed in the white individuals. Other platelet agonists, including PAR1, were not different between the groups. A second study identified two additional polymorphisms that change amino acids in PAR4 at positions 120 (Ala/Thr) and 296 (Phe/Val) (Edelstein et al. 2014). The polymorphism at 120 is common and is distributed by race. PAR4-120A exhibited a lower reactivity and was found in 81 % of white individuals compared to 37 % of black individuals. In contrast, PAR4-120T was hyperreactive to agonists, resistant to a PAR4 antagonist, and found in 63 % of blacks compared to 19 % of whites (Edelstein et al. 2014). The frequency of Val at 296 was low and had low reactivity regardless of the amino acid at 120 suggesting that it is a dominant negative receptor. The mechanism by which the PAR4 variants elicit their distinct response to affect platelet function is not known. These polymorphisms may change the interaction of PAR4 with the membrane, allosterically alter ligand binding, or influence the transition of the receptor to an active state (Isberg et al. 2014). Structural studies examining the differences between the PAR4 sequence variants are necessary to determine the molecular basis for the differences in reactivity.

Structural Studies on PARs

The platelet field has benefited from the recent advances in membrane protein crystallography (Salon et al. 2011; Zhang et al. 2012a, 2014a, b). Included in this list is the high-resolution crystal structure of PAR1 bound to the antagonist vorapaxar (Zhang et al. 2012a). The general overall structure was similar to many other GPCRs. However, when the PAR1 structure is compared to other class A GPCRs, transmembrane helix 7 was structurally similar to activated receptors, which is surprising for an antagonist bound receptor. Complementary studies are needed to determine if this is a unique feature of PAR1 or if it is specific to the conformation induced by the antagonist vorapaxar. The experimental constraints and sequence modifications required for GPCR crystallography have thus far have prevented a detailed structural analysis of the tethered ligand mechanism (Salon et al. 2011). Recently, Alsteens and colleagues developed a modification of atomic force microscopy to probe the ligand binding site of PAR1 and determined the free-energy landscape (Alsteens et al. 2015). Based on these studies, the authors proposed a two-step binding mechanism where the ligand first interacts in a low-affinity mode and then progresses to a high-affinity mode. Further studies are necessary to determine if the two-step mechanism is shared across the PAR family and how these observations are linked to the NMR studies with the PAR1 exodomain (Seeley et al. 2003). Since PARs are grouped together by their common activation mechanism, it is tempting to use PAR1 as the preferred model to gain structural insight for other PARs. However, PARs share no more sequence identity between family members than other GPCRs in general (34–41 %). There will undoubtedly be specific information regarding activation mechanism that is unique to each PAR as more structural and biophysical data become available.

Species Differences in PARs Expression in Platelets

Animal models are widely used for preclinical studies to examine platelet function and pharmacology in vivo. There are important differences in how platelets respond to thrombin between species (Connolly et al. 1994; Derian et al. 1995). Comparative ultrastructural and functional studies of platelets show differences in the open canalicular system (OCS), cytoskeletal proteins, and regulatory proteins, which may impact the kinetics of dense granule release (Choi et al. 2010; Gruba et al. 2015). The major contributor to species-specific responses of platelets to thrombin is the repertoire of PARs expressed on their platelets.

PAR4 is expressed on the platelets of most species, whereas PAR1 and PAR3 expression is more limited. PAR1 is expressed on platelets from human, monkey, and guinea pig (Fig. 6). PAR3 is expressed on platelets from mouse, rabbit, rat, and dog (Connolly et al. 1994; Derian et al. 1995). Monkey and guinea pig are the only animal models that express both PAR1 and PAR4 on their platelets.

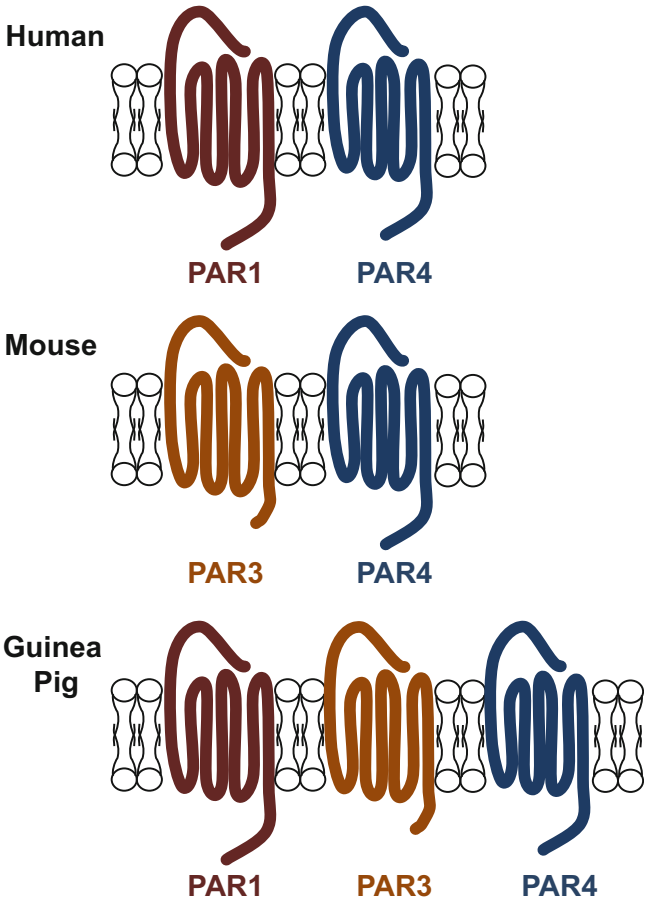

Fig. 6 Species differences in PAR expression on platelets from commonly used preclinical animal models

However, guinea pigs also express PAR3, which makes thrombin signaling more complicated in this animal model (Andrade-Gordon et al. 2001). Platelets from guinea pigs respond tenfold less to SFLLRN in aggregation experiments despite 80 % sequence identity between human PAR1 and guinea pig PAR1 (Kinlough-Rathbone et al. 1993). The high expression of PAR1, PAR3, and PAR4 influences pharmacology experiments. For example, the PAR1 inhibitor RWJ-58259 showed no significant antithrombotic effect in guinea pig thrombosis models in vivo (Andrade-Gordon et al. 2001). Taken together, guinea pigs have limitations as an animal model that need to be considered when evaluating PAR antagonists as potential antithrombotic drugs for humans. Genetically altered mice have been widely used in thrombosis studies to determine the contributions of platelet proteins in vivo. A mouse model with "humanized" PAR expression on their platelets would be an important tool for examining the specific individual contributions of PAR1 and PAR4 in vivo. Mice expressing PAR1 and PAR4 on their platelets would also serve as a convenient preclinical model for antiplatelet agents. To date, these efforts have been unsuccessful (Arachiche et al. 2014).

Take-Home Messages

- Thrombin is a potent platelet agonist that signals via proteolytic cleavage of protease activated receptors (PARs).
- PARs are G-protein-coupled receptors that signal through G_q and $G_{12/13}$ in platelets.
- PAR expression on platelets varies among species; human platelets express PAR1 and PAR4.
- PAR1 and PAR4 have distinct activation and signaling kinetics, which are influence via cooperation between PAR family members.

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