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Review

Kallikreins and proteinase-mediated signaling: proteinase-activated receptors (PARs) and the pathophysiology of inflammatory diseases and cancer*

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Abstract

Proteinases such as thrombin and trypsin can affect tissues by activating a novel family of G protein-coupled proteinase-activated receptors (PARs 1–4) by exposing a 'tethered' receptor-triggering ligand (TL). Work with synthetic TL-derived PAR peptide sequences (PAR-APs) that stimulate PARs 1, 2 and 4 has shown that PAR activation can play a role in many tissues, including the gastrointestinal tract, kidney, muscle, nerve, lung and the central and peripheral nervous systems, and can promote tumor growth and invasion. PARs may play roles in many settings, including cancer, arthritis, asthma, inflammatory bowel disease, neurodegeneration and cardiovascular disease, as well as in pathogen-induced inflammation. In addition to activating or disarming PARs, proteinases can also cause hormone-like effects via PAR-independent mechanisms, such as activation of the insulin receptor. In addition to proteinases of the coagulation cascade, recent data suggest that members of the family of kallikrein-related peptidases (KLKs) represent endogenous PAR regulators. In summary: (1) proteinases are like hormones, signaling in a paracrine and endocrine manner via PARs or other mechanisms; (2) KLKs must now be seen as potential hormone-like PAR regulators *in vivo*; and (3) PAR-regulating proteinases, their target PARs, and their associated signaling pathways appear to be novel therapeutic targets.

Keywords: arthritis; coagulation cascade; colitis; pain; thrombin; trypsin.

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Introduction

Proteinases as hormone-like signal messengers

Proteinases have long been known for their ability to function in the digestive tract and to generate active peptides from polypeptide hormone precursors. What is not as well known, however, is that work in the mid-1960s led to the surprising observation that proteinases such as trypsin, pepsin and chymotrypsin could mimic the actions of insulin to promote glycogen formation in a rat diaphragm preparation (Rieser and Rieser, 1964; Rieser, 1967). Subsequent publications in the early 1970s showed that in isolated adipocyte preparations, trypsin, like insulin, could simultaneously stimulate glucose oxidation and inhibit lipolysis (Kono and Barham, 1971). Furthermore, it was observed that, like insulin, proteinases, including the coagulation cascade serine proteinase thrombin, are anabolic agents that can stimulate mitogenesis in cultured cell systems (Burger, 1970; Sefton and Rubin, 1970; Chen and Buchanan, 1975).

Proteinase-mediated signaling

Identified in work that began in the late 1980s, mechanisms responsible for the cellular actions of a number of proteinases have now come into sharper focus. For instance, the insulin-like actions of trypsin can be attributed to activation of the insulin receptor via truncation of the receptor's α-chain, which acts as a negative regulator of the receptor's catalytic activity (Shoelson et al., 1988). Nonetheless, the cellular actions of trypsin and other serine proteinases have now been identified as due to the regulation of a unique family of G protein-coupled receptors, the proteinase-activated receptors, referred to by the International Union of Pharmacology-approved acronym PARs (Hollenberg and Compton, 2002). In addition to regulating PAR activity, proteinases can regulate tissue function by other mechanisms that are discussed here. This overview aims to summarize both PAR-related and unrelated mechanisms by which serine proteinases can regulate cellular signal transduction and to summarize our preliminary data pointing to a tissue regulatory and inflammatory role for the kallikrein-related peptidase (KLK) family of serine proteinases. The KLK-mediated effects that we describe can be attributed to their ability to regulate PAR activation. Our data support the working hypothesis that proteinases, including human KLKs, must now be considered as important 'hormonal' regulators of inflammation, nociception and cardiovascular function, thus representing new therapeutic targets for

Table 1 Receptor-selective PAR-activating peptides and receptor-inactive control peptides.

Receptor	PAR-AP	Standard inactive control peptide
PAR_1	TFLLR-NH_2	**FT**LLR-NH_2
PAR_2	SLIGRL-NH_2; 2furoyl-LIGRLO-NH_2	**LS**IGRL-NH_2; 2-furoyl-OLRGIL-NH_2
PAR_3	–	–
PAR_4	AYPGKF-NH_2	**YA**PGKF-NH_2

Reversal of the sequence of only the first two amino acids of an activating peptide (shown in bold) results in a sequence that cannot activate PARs. PAR_3 is not activated via PAR-APs; its tethered ligand sequences activate PARs 1 and 2.

the treatment of vascular, neoplastic and inflammatory diseases.

Proteinase-activated receptors: discovery and signaling mechanisms

Searching for the 'thrombin receptor'

Although by the 1970s the observations outlined above showed that serine proteinases can regulate cell function by mimicking the action of insulin and a variety of mitogens, a systematic approach to identify the target receptors was not undertaken until the early 1990s. The impetus for the discovery of membrane receptors that can be activated by proteinases came from the search for the 'receptor' responsible for the actions of thrombin on platelets. Early studies of thrombin binding to cells did not succeed in identifying that receptor, although a number of interesting cell surface non-receptor binding sites for thrombin were identified. In contrast, an expression cloning approach using mRNA derived from thrombin-responsive fibroblasts and platelet precursor cells led two laboratories to independently clone the 'thrombin receptor' (Rasmussen et al., 1991; Vu et al., 1991). Unexpectedly, the cloned sequence revealed that the receptor triggered by thrombin is a member of the G protein-coupled receptor (GPCR) superfamily. It is now known that the PAR family comprises four members (PARs 1–4: Tables 1 and 2), each with a distinct pharmacology and physiology (Hollenberg and Compton, 2002; Ossovskaya and Bunnett, 2004; Coughlin, 2005; Steinhoff et al., 2005).

Mechanisms of receptor regulation

In exploring the mechanisms by which thrombin activates signaling via its GPCR target, Vu et al. (1991) discovered two very remarkable properties (Figure 1). (1) The proteinase unmasks a cryptic N-terminal extracellular receptor sequence that, remaining tethered, binds to the extracellular receptor domains to activate signaling (lower portion, left panel, Figure 1). This 'tethered ligand' (TL) mechanism is unique for this family of GPCRs. (2) Synthetic peptides based on the revealed TL sequence in the absence of receptor proteolysis are able to activate the receptor and trigger cell signaling (middle panel, Figure 1). Each of these two characteristics confers a 'special' pharmacology on the PARs. First, since activation involves tryptic cleavage of the N-terminal sequence to unmask the TL, a variety of serine proteinases can in principle serve as receptor 'agonists'. Second, as illustrated in the right-hand panel of Figure 1, if a proteinase cleaves downstream from a PAR TL sequence, the receptor becomes 'disarmed', such that an activating proteinase can no longer generate a signal. Nonetheless,

Table 2 Potential physiological roles for PARs.

PAR involved	Potential role	Comment
1, 3 and 4	Platelet activation, hemostasis	PARs regulate both secretion and aggregation; PAR_1 and PAR_4 can play separate roles
1, 2 and 4	Modulation of endothelial cell function	Release of NO, von Willebrand factor; increased neutrophil adherence; cell migration
1, 2 and 4	Modulation of vascular smooth muscle function	Activation of contractility; angiogenesis?
1, 2 and 4	Modulation of intestinal function	Regulation of motility (smooth muscle), secretion (epithelial cell) and inflammation (enteric neuronal cells)
1, 2 and 4	Modulation of renal vascular function	Regulation of flow and afferent arteriolar function
1 and 2	Modulation of CNS neuronal and astrocyte function	Upregulation of PARs in the setting of CNS inflammation
2	Inflammatory response to joint injury	Key role for PAR_2 in arthritis
1 and 2	Promoting tumor cell growth and metastasis	Both PAR_1 and PAR_2 may play roles, activated by tumor-derived serine proteases and matrix metalloproteinases (e.g., MMP-1)
2	Skin pigmentation	Proteinase inhibition affects skin pigmentation; involvement of PAR_2 in ethnic skin color phenotypes
1, 2 and 4	Hyperalgesia and analgesia	PAR activation can increase (PAR_2) or decrease (PAR_1, PAR_4) pain sensation

This Table is meant only as an introductory overview of the potentially broad tissue impact of PAR function. For a more comprehensive overview of the possible physiological roles that PARs may play, see the special issue volumes *59* (issue 4) and *60* (issue 1) of Drug Development Research available on the website http://www.inflammation-calgary.com.

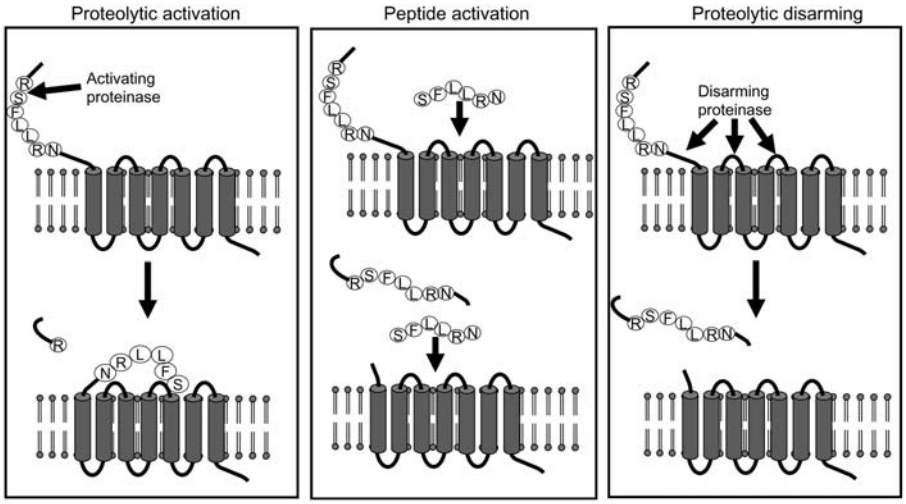

Figure 1 Model of activation and disarming of PAR_1.
The scheme shows the proteolytic activation of human PAR_1 by the unmasking of its tethered ligand (TL) sequence. Once revealed by an activating proteinase (e.g., thrombin), the TL sequence binds to the receptor extracellular domain to trigger activation (bold arrow, lower portion of left-hand panel). Alternatively (middle panel, top portion), a synthetic peptide with a sequence representing the first five or six amino acids of the TL domain can activate the receptor without the need for proteolysis (bold arrow). As shown on the right, a proteinase that cleaves downstream of the tethered ligand sequence can 'disarm' the receptor, preventing activation by another enzyme (right panel, lower portion). Nonetheless, the disarmed receptor can still respond to a PAR-activating peptide (middle panel, lower portion).

the disarmed receptor can still respond to an activating peptide (lower portion, middle panel, Figure 1). Thus, in principle, each PAR can have multiple proteinase activating 'agonists' and disarming 'antagonists'.

Activation versus disarming PARs

In selected settings in which target cells express multiple PARs (e.g., both PAR_1 and PAR_4 are expressed in human platelets and both PAR_1 and PAR_2 are expressed in the vascular endothelium of many species), the ability of a proteinase to silence an individual PAR by disarming may be as relevant physiologically as is PAR activation. For instance, in cultured HEK-293 cells that express both PAR_1 and PAR_2, relatively low trypsin concentrations (0.5–1.5 nM or 0.25–0.75 U/ml) not only activate a PAR_2 signal (elevated intracellular calcium), but also simultaneously remove the activation sequence from PAR_1, thus preventing its activation by thrombin (Kawabata et al., 1999). Furthermore, in lung inflammation, in which a serine proteinase like leukocyte elastase may be present, PAR_2 can be disarmed to silence its potential physiological function (Dulon et al., 2003). Even when disarmed, however, a PAR remaining at the cell surface can still respond to a PAR-activating peptide (Figure 1).

Discovering pathophysiological roles for PARs: use of PAR-activating peptides, PAR-null mice and PAR blockade in model systems

PAR-activating peptides as probes for PAR function *in vivo*

The ability of synthetic peptides derived from the TL sequences of the PARs to mimic activation by proteolysis (Vu et al., 1991) provided an invaluable key for understanding the many physiological roles that the PARs can play. By using PAR-activating peptides as a stimulus, it was possible to circumvent the complexity of interpreting a response triggered by an enzyme such as trypsin, which can affect more than one PAR simultaneously and may also target other receptors. From peptide structure-activity studies, it was possible to design PAR-selective peptide agonists (PAR-APs) and receptor-inactive control peptides (Table 1) that have proved of considerable value for studies with cultured cells and tissue targets both *in vitro* and *in vivo*. As summarized in the following sections, results with PAR-APs, PAR-null mice and receptor antagonists indicate that PARs play an important role in the pathophysiology of diseases ranging from inflammation and pain to cardiovascular dysfunction, degenerative diseases of the central nervous system (CNS) and cancer. For an overview of articles dealing with PARs and their potential impact on physiological function, the reader is referred to the special issues of Drug Development Research [volumes 59(4) and 60(1), 2003] available on the website http://www.inflammation-calgary.com. Further information on PARs can be found in more recent reviews (Ossovskaya and Bunnett, 2004; Coughlin, 2005; Steinhoff et al., 2005; Bushell, 2007; Hansen et al., 2007; Moffatt, 2007; Ramachandran and Hollenberg, 2008).

PARs, inflammation, neuronal responses and nociception

In the setting of inflammation, it is known that mast cells can release a variety of proteinases that contribute to the overall process. Based on the hypothesis that mast cell proteinases released at a site of inflammation might activate PARs, we first used PAR-APs to assess the potential impact of PAR activation in a paw edema model of inflammation compared to the inflammatory response

generated by intraplantar administration of a known PAR_2 activator, trypsin. In that model, administration of small doses of either a PAR_2- or PAR_1-activating peptide caused marked swelling and leukocyte infiltration comparable to that caused by either trypsin or thrombin (Vergnolle et al., 1999a; Vergnolle et al., 1999b). PAR_2-activating peptides were also found to induce intestinal inflammation (Cenac et al., 2002). Early on, it was also observed that functional PAR_2 and PAR_1 could be localized on neurons from the myenteric plexus of guinea-pig small intestine in primary culture; the neuronal cells responded to activation by PAR_1- and PAR_2-activating peptides, with an increase in intracellular calcium (Corvera et al., 1999). Together, these two sets of observations led to the discovery that the inflammatory response triggered by PAR_1 and PAR_2 involves a neurogenic mechanism (Steinhoff et al., 2000; de Garavilla et al., 2001).

One of the more impressive links between PAR activation and inflammation is in the setting of arthritis. For example, in a murine arthritis model, PAR_2 plays an essential role (Ferrell et al., 2003). Furthermore, by targeting and blocking PAR_2 activation, it is possible to diminish inflammation in a murine arthritis model (Kelso et al., 2006). Thus, a focus on PAR_2 and its role in arthritis has substantial therapeutic implications. Administration of a PAR_4-activating peptide also causes the formation of edema and leukocyte recruitment in a rat paw model of inflammation. However, in contrast to PAR_1 and PAR_2, the inflammation caused by PAR_4-activating peptide is not dependent on a neurogenic mechanism (Hollenberg et al., 2004; Houle et al., 2005).

Given that PARs on nerve cells are partly responsible for triggering inflammation (Steinhoff et al., 2000), a logical question to pose was whether PARs are also involved in sensing pain. Indeed, nociception does involve PAR activity (Vergnolle et al., 2001a,b; Asfaha et al., 2002; Vergnolle, 2004). Since PARs are widely distributed on neurons and their associated cells, such as astrocytes, both in the CNS and peripheral nervous system, it is expected that neuronal PARs play a widespread physiological role. As an example, upregulation of PAR_1 occurs in the CNS in HIV encephalitis (Boven et al., 2003). Furthermore, PAR_2 appears to play a neuroprotective role in HIV infection (Noorbakhsh et al., 2005), but a deleterious role in an animal model of multiple sclerosis (Noorbakhsh et al., 2006).

PARs and cardiovascular function

When PAR_2 was fortuitously cloned in the course of a search of a mouse liver-derived genomic library with probes for the bovine substance K receptor (Nystedt et al., 1994), its function was not yet known. However, peptide structure-activity studies with a rat vascular preparation pointed to the presence of functional PAR_2 in the endothelium (Al-Ani et al., 1995). Thus, an isolated rat aorta tissue preparation provided one of the first bioassay systems that enabled us to predict a role for PARs in regulating vascular function (Muramatsu et al., 1992; Al-Ani et al., 1995; Saifeddine et al., 1996). Using isolated rat aorta tissue, it has been possible to document the ability of PAR_2, as well as PAR_1, to activate endothelium-dependent nitric oxide (NO)-mediated vasorelaxation. In an endothelium-free vessel, activation of PAR_1, either by a PAR_1-selective activating peptide such as TFLLR-NH_2 or by thrombin, causes prompt vasoconstriction, whereas PAR_2 activation has no such effect. In an isolated kidney preparation, both PAR_1 and PAR_2 can have an effect on perfusion, with PAR_1 activation causing a marked decrease in flow similar to that induced by angiotensin and PAR_2 acting as a vasodilator to increase flow (Gui et al., 2003). Thus, in certain settings, PAR_1 and PAR_2 may play a counter-regulatory role. Although PAR_2 activation primarily leads to a NO-mediated relaxation in conduit vessels such as the aorta, vasodilatation in resistance vessels or in renal afferent arterioles caused by PAR_2 activation is mediated not only by NO, but also by as yet unidentified endothelium-derived relaxing factors (EDHFs) (McGuire et al., 2004; Wang et al., 2005). The impact of PAR_4 activation on vascular function is not yet clear, except that it appears to play a potential role in endothelium-leukocyte interactions (Vergnolle et al., 2002).

Another potential role for PAR_2 in the context of cardiovascular disease may occur in ischemia-reperfusion, in which upregulation of PAR_2 can promote vasodilatation (Napoli et al., 2000). It has also been found that PAR_2 is increased in human coronary atherosclerotic lesions (Napoli et al., 2004). In a mouse model of type 1 diabetes, it has been observed that the vasodilatory response to PAR_2 stimulation increased, whereas there was a progressive reduction in the response to acetylcholine (Roviezzo et al., 2005). The distinct effects on blood pressure and heart rate upon activating either PAR_1 (both hypotension and tachycardia) or PAR_2 (hypotension only, without an effect on heart rate) have been unequivocally established using mice deficient in either PAR_1 or PAR_2 (Damiano et al., 1999). Thus, there appears to be a generalized role for PARs in the setting of cardiovascular pathophysiology.

PARs, cancer and metastasis

Since the 1990s it has been suggested that the coagulation system in general and thrombin specifically via PAR_1 may play an important role in tumor growth and metastasis (Nierodzik et al., 1998; Henrikson et al., 1999). Not only might thrombin facilitate cell migration to metastatic sites, but it has been known since the mid-1970s that the enzyme itself is a particularly potent mitogen for normal and tumor-derived cells (Chen and Buchanan, 1975), presumably acting via PAR_1. A clear link has been made between PAR_1 expression and the ability of mammary tumor-derived cells to migrate in culture through a reconstituted basement membrane (Even-Ram et al., 1998). The ability of PAR_1 to play a role in tumor metastasis and invasion is underlined by the ability of tumor-derived matrix metalloproteinase-1 to activate the receptor and drive the process of migration and metastasis of breast carcinoma cells in a xenograft (Boire et al., 2005). A comparable role for PAR_2 in the setting of cancer is expected (Shi et al., 2004). Given the information provided in the previous sections, it is clear that in addition to contributing to the growth and metastasis of tumor cells, PARs potentially play a role in a wide variety

of pathophysiological processes, as summarized in Table 2.

From the information presented in the above sections, the unifying hypothesis that can be put forward is that PARs play a key role in the body's innate immune defense system as a primary trigger of the inflammatory response and pain sensation due to tissue injury or remodeling caused by pathogenic processes. As outlined above, this hypothesis is strongly supported by the striking resistance of PAR_2-deficient mice to adjuvant-induced arthritis (Ferrell et al., 2003) and by the ability to diminish arthritis in a murine model by antagonizing PAR_2 (Kelso et al., 2006).

Identifying PAR-regulating proteinases that may play a role *in vivo*

Although it has been possible using a pharmacological approach to evaluate the potential effects of a number of proteinases to either activate or disarm PARs (e.g., Kawabata et al., 1999), it is a challenge to establish the precise enzymes that might be responsible for regulation of PARs (or other proteinase signaling targets) *in vivo*. The following sections deal with this issue.

Coagulation pathway enzymes

Since PAR_1, PAR_3 and PAR_4 were discovered as a result of the search for the cellular target of thrombin, there is little doubt that this enzyme represents a key activator for PAR_1 and PAR_4 *in vivo*, with the participation of PAR_3 as a co-factor. This conclusion is supported by a number of approaches, including the use of PAR-null animals and PAR antagonists (Coughlin, 2005). However, it was not initially appreciated that other coagulation-pathway enzymes could target PAR_2, as does the tissue factor-VIIa/Xa complex (Ruf et al., 2003; Versteeg and Ruf, 2006), and that in certain circumstances factor Xa can activate PAR_1 (Blanc-Brude et al., 2005; Bhattacharjee et al., 2008). In the counter-regulatory clotting cascade pathway, both plasmin and activated protein C (APC) can in principle affect the PARs. The action of plasmin on PARs is complex, in that this fibrinolytic enzyme can both activate and disarm PAR_1 (Kimura et al., 1996; Kuliopulos et al., 1999) and can activate PAR_4 (Quinton et al., 2004). This action of plasmin may be complemented by its ability to generate monocyte-macrophage cell signals by cleaving the annexin A2 heterotetramer (Laumonnier et al., 2006; Li et al., 2007). APC, via its endothelial adsorption site and a targeted interaction involving its exosite domain, can activate PAR_1 (Riewald et al., 2002; Yang et al., 2007). Thus, *in vivo*, enzymes of the coagulation cascade that are known to be activated in a number of settings can be considered as physiological regulators of PAR activity.

Proteinases of the gastrointestinal tract

Upon its discovery, PAR_2 was found to be particularly sensitive to activation by trypsin, whereas the receptor was not readily activated by thrombin (Nystedt et al., 1994). When PAR_2 was subsequently localized at the apical membrane of rat enterocytes, it was proposed that trypsin present in the intestinal lumen may represent a physiological regulator of PAR_2 at this site (Kong et al., 1997). In a rat model of colitis, considerably elevated levels of serine proteinase activity were observed in colon tissue, as well as in tissues from ulcerative colitis patients (Hawkins et al., 1997). Furthermore, studies of intestinal biopsy samples from humans with inflammatory bowel syndrome revealed the release of serine proteinases that can activate PAR_2 (Cenac et al., 2007). The release of luminal serine proteinases that can activate PAR_2 has also been documented in a murine model of infectious colitis. In that study, not only were trypsin family members identified in the luminal contents by mass spectral analysis, but the administration of a trypsin inhibitor was able to mitigate the inflammatory process triggered by the infection (Hansen et al., 2005). Thus, it appears that endogenous tryptic-like colonic proteinases can activate PAR_2 known to be present on enterocytes. Since a variety of other proteinases are also generated in the colon in inflammatory disease (e.g., cathepsins, metalloproteinases), it is highly likely that by either activating or disarming the PARs, these enzymes play important inflammatory roles in colitis via the PARs (Vergnolle, 2005).

Tumor-derived proteinases: potential role for KLKs

Although much attention has been focused on the potential role of matrix metalloproteinases in cancer spread (Shapiro, 1999), serine proteinases are also released in a tumor environment. The family of KLKs, of which the best known is prostate-specific antigen (PSA) or kallikrein-related peptidase 3 (KLK3), has attracted substantial attention as a group of serine proteinases linked to cancer-associated pathophysiology (Borgoño and Diamandis, 2004; Borgoño et al., 2004). The human KLKs, which are secreted as inactive zymogens, can exhibit either trypsin (twelve family members) or chymotrypsin-like (three family members) activity upon proteolytic activation (Borgoño et al., 2004). Several members of the family have been shown to possess clinical value as biomarkers for cancer diagnosis and prognosis (Borgoño and Diamandis, 2004). In this regard, KLK3 is a valuable marker for prostate cancer diagnosis, together with another member of the family, KLK2 (Becker et al., 2001).

Although the KLKs are differentially expressed in cancer and other diseases, in relation to normal tissues (Borgoño and Diamandis, 2004; Borgoño et al., 2004; Shaw and Diamandis, 2007) there is as yet little information about the functions that these enzymes might play *in vivo*. Several *in vitro* studies have identified potential substrates implicated directly in carcinogenesis, for example, extracellular matrix proteins, kininogens, pro-urokinase-plasminogen activator (pro-uPA), growth factor precursors and their binding proteins. Other KLKs may also be targets of kallikrein proteolysis during cancer progression (Borgoño and Diamandis, 2004; Borgoño et al., 2004). By generating active peptides such as the bradykinins, KLK1 can influence cell function by activating the bradykinin receptors B1 and B2. Furthermore, the release of a variety of active peptides from precursors, in addition to the kinins, represents one way in which the KLKs can

affect tissues (Borgoño and Diamandis, 2004; Borgoño et al., 2004). We have suggested that although such substrates (e.g., extracellular matrix, pro-uPA, growth factor precursors) can explain some of the physiological actions of KLKs, in the setting of cancer, where a direct effect of KLKs on cell function is of importance, many of their biological targets are still unknown. Thus, in our recent work we have hypothesized that the tissue kallikreins can signal to cells by cleavage and activation of PARs (Oikonomopoulou et al., 2006a,b).

To test this hypothesis, we have used a variety of methods, including analysis of KLK-mediated cleavage of synthetic PAR N-terminal derived peptides, calcium signaling subsequent to KLK-triggered activation or deactivation of PARs in cultured cells and isolated platelets, vascular contraction and relaxation bioassays stimulated by KLKs and *in vivo* measurements of murine paw edema in response to KLKs (Oikonomopoulou et al., 2006a,b).

We found that all three of the KLKs we evaluated (KLK5, 6 and 14) can cleave the synthetic peptides representing the cleavage-activation sequences of PAR_1, PAR_2 and PAR_4 at sites that would potentially result in either receptor activation or disarming (Oikonomopoulou et al., 2006a). All three KLKs also cause increases in intracellular Ca^{2+} in target cells and platelets by activating one or more of the PARs. Importantly, there are distinct differences between KLK5, 6 and 14 in terms of their selective actions on each of PAR_1, PAR_2 and PAR_4. For instance, KLK14 can activate PAR_2 and PAR_4. However, depending on its concentration, KLK14 can either activate (high concentration) or disarm (low concentration) PAR_1, thereby preventing its activation by thrombin (Oikonomopoulou et al., 2006a). In contrast to KLK14, KLK5 and 6 preferentially activate PAR_2, leaving PAR_4 intact. Thus, *in vivo*, like thrombin, KLK14 is a potential regulator of platelet function, but via a mechanism different from that of thrombin, with predominant activation of platelet PAR_4, whereas thrombin preferentially activates PAR_1. In contrast, neither KLK5 nor KLK6 can activate platelets. Like trypsin, all three KLKs we tested (KLK5, 6 and 14) can activate rat aorta vascular endothelial PAR_2 to cause NO-dependent relaxation (Oikonomopoulou et al., 2006a). Comparable results would be expected for human vascular tissue. Thus, *in vivo*, KLKs may be potential regulators of cardiovascular function via PARs. Also like trypsin and tryptase, we have shown that KLK14 can cause an inflammatory paw edema response when administered *in vivo* (Oikonomopoulou et al., 2006b). Further work is required to evaluate the potential inflammatory and nociceptive roles that, like trypsin, KLKs may play *in vivo*.

Given the biological actions we have observed for KLK5, 6 and 14, we suggest that these enzymes, when produced by tumors, may, like thrombin, promote tumor cell growth and invasion (Even-Ram et al., 1998). Because of their ability to regulate tissues via the PARs, their wide expression in regions where trypsins may not be produced and their possible participation in enzymatic cascades, the KLKs represent important potential physiological regulators of tissue function *in vivo*. The challenge that remains is to determine whether or not the high levels of KLKs detected by immunoassay in tumor-derived or body fluid samples represent enzymatically active species.

Therapeutic opportunities and proteinase-mediated PAR signaling

Targeting PARs

An initial stimulus for the discovery of PAR_1 was the likelihood that it would be possible to prevent platelet activation by blocking the thrombin receptor without blocking other effects of thrombin, such as coagulation. Thus, the bleeding problems induced by coumadin-related anticoagulants could be avoided. Similarly, the inflammatory role discovered for PAR_2 in the setting of arthritis (Ferrell et al., 2003) led to proof of the principle that blocking PAR_2 can diminish joint inflammation in a murine arthritis model (Kelso et al., 2006). In human and rodent platelets, PAR_4 also plays an important regulatory role. Thus, PAR_4 antagonists may eventually prove to be of therapeutic benefit (Covic et al., 2002), in particular because the actions of PAR_1 and PAR_4 differ in human platelets. In view of the importance of developing PAR antagonists for therapeutic purposes, significant progress has been made in developing PAR_1-targeted antagonists that exhibit therapeutic potential in selected settings (Maryanoff et al., 2003; Chackalamannil et al., 2005; Camerer, 2007). Unfortunately, the development of a high-potency PAR_2 antagonist has been elusive and the PAR_4 antagonists developed so far are peptides of relatively low potency (Hollenberg and Saifeddine, 2001; Covic et al., 2002), so that their potential clinical applicability is quite restricted.

Blocking proteinases

The design of high-affinity, enzyme-selective proteinase inhibitors can lead to very useful therapeutic agents, such as angiotensin-converting enzyme (ACE) inhibitors (Cushman and Ondetti, 1999). The design of such agents depends on a detailed knowledge of enzyme mechanism, crystallographic information and a lead structure often derived from naturally occurring inhibitors, such as snake venom-derived peptide ACE inhibitors. In the case of serine proteinases such as the KLKs, it is possible that modified naturally occurring serpins will provide an avenue for developing clinically useful selective inhibitors (Felber et al., 2006). In terms of PAR regulation, however, the number of serine proteinases that can potentially regulate signaling by either activation or disarming is large, and the design of enzyme inhibitors, apart from those targeted to thrombin, may prove very difficult. For instance, inhibitors designed to target tryptase, such as nafamosatat, are also able to block a variety of other serine proteinases with considerable potency. Thus, the design of potent and selective proteinase inhibitors to attenuate PAR regulation will very likely prove quite challenging. Nonetheless, by inhibiting proteolytic activity in appropriate settings and by blocking PARs, it is possible to envision novel therapeutic opportunities that may prove useful in diseases such as pancreatitis and arthritis that have been very difficult to treat to date. This dual

approach, blocking both PARs and proteinases, also has merit in terms of the treatment of tumor growth and invasion.

Summary

We have provided an overview of the discovery of the PAR family and the approaches that have been used, primarily with receptor-selective PAR-APs, PAR-null mice and a limited number of receptor antagonists, to determine the potential role that PARs may play *in vivo*. The data suggest prominent roles for PARs in a number of areas, including cancer, arthritis, asthma, inflammatory bowel disease, neurodegeneration and cardiovascular disease, as well as in pathogen-induced inflammation. Serine proteinases of the coagulation cascade, as well as trypsin family members, can be viewed as likely regulators of PARs *in vivo*, and we suggest that the trypsin-related members of the KLK family may also represent physiological PAR regulators. Moreover, other enzyme families, including metalloproteinases and cathepsins, can also be envisioned as potential PAR regulators (either activators or inactivators) *in vivo* (Antalis et al., 2007). Furthermore, as with trypsin, serine proteinases and members of other proteinase families very likely regulate cell signaling via non-PAR mechanisms, such as activation of the insulin receptor. Thus, proteinases can now be generally regarded as 'hormone-like' messengers that can signal via PARs or other mechanisms. As a result, PARs, their activating proteinases and their associated signal transduction pathways can now be considered as attractive therapeutic targets.

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