Proteinases, Their Extracellular Targets, and Inflammatory Signaling

Rithwik Ramachandran, Christophe Altier, Katerina Oikonomopoulou, and Morley D. Hollenberg

ABSTRACT

I. Introduction .................................................. 1111

II. Classification and Overview of Mechanisms Regulating Proteolytic Enzymes ........ 1112

III. Proteinase-Mediated Signaling: Molecular Targets that Trigger the Response ....... 1114

A. Igniting the Inflammatory Response via Proteolytic Signaling ................................ 1114

B. Molecular Targets .............................................................................................................. 1114
   1. Proteolytic Cascades that Generate Active Inflammatory Polypeptides: The
      Coagulation, Complement, Kallikrein-kininogen, and Tissue Kallikrein-kallikrein-
      Related Peptidase Systems ............................................................................................... 1114
   2. Complement and Other Interconnected Proteolytic Cascades ..................................... 1114
   4. Kallikrein-Related Peptidases and the Production of Inflammation-Related Peptides 1116
   5. Generation of Kinins by Kallikrein-Related Peptidase 1 ............................................... 1117
   6. Interplay between Kallikrein-Related Peptidases, Coagulation Proteinases, and the
      Complement Cascade ....................................................................................................... 1117

C. Hormone-like Signaling by Proteinases: Mimicking the Action of Insulin and
   Mitogenic Polypeptides ......................................................................................................... 1117

D. Proteinase-Activated G Protein-Coupled Receptors: A New Paradigm for G Protein-
   Coupled Receptor Signaling ............................................................................................... 1118
   1. “Tethered Ligand” Receptor Domains Responsible for Proteinase-Activated
      Receptor Activation ......................................................................................................... 1118
   2. Synthetic Receptor-activating Peptides Based on the Tethered Ligands ..................... 1119
   3. “Noncanonical” Proteinase-Activated Receptor Tethered Ligands Revealed by
      Proteinases other than Thrombin and Trypsin ............................................................... 1119
   4. Regulation of Proteinase-Activated Receptor Signaling: Desensitization,
      Internalization, and Intracellular Targeting .................................................................... 1120
   5. Proteinase-Activated Receptor 1 .................................................................................. 1122
   6. Proteinase-Activated Receptor 2 .................................................................................. 1123
   7. Proteinase-Activated Receptors 3 and 4 ...................................................................... 1123
   8. Effectors that Mediate Proteinase-Activated Receptor Signaling ................................ 1123
   9. Posttranslational Modifications of Proteinase-Activated Receptors and Proteinase-
      Activated Receptor Function ........................................................................................... 1123

E. Endogenous Proteolytic Regulators of Proteinase-Activated Receptor Function and
   Proteinase-Activated Receptor Signaling ............................................................................ 1124
   1. Coagulation Cascade Enzymes ..................................................................................... 1124

Work in the authors’ laboratories that underpins the information in this review is funded by operating grants from the Canadian Institutes for Health Research (M.D.H., C.A.), Prostate Cancer Canada (M.D.H., C.A., R.R.), the Natural Sciences and Engineering Research Council of Canada (C.A.), and The Calgary Motorcycle TELUS Ride for Dad and the Prostate Cancer Fight Foundation (M.D.H.).

Address correspondence to: Morley D. Hollenberg, Department of Physiology & Pharmacology, University of Calgary Cumming School of Medicine, 3330 Hospital Drive NW, Calgary AB, Canada T2N 4N1. E-mail: mhollenb@ucalgary.ca
dx.doi.org/10.1124/pr.115.010991.
Proteolytic ion channel regulation. These mechanisms are common with adhesion-G protein-coupled receptors (ADGRs); and by protein-coupled receptors (GPCR), with a mechanism in the extracellular microenvironment can regulate cell unmasking.

Abstract—Given that over 2% of the human genome codes for proteolytic enzymes and their inhibitors, it is not surprising that proteinases serve many physiologic-pathophysiologic roles. In this context, we provide an overview of proteolytic mechanisms regulating inflammation, with a focus on cell signaling stimulated by the generation of inflammatory peptides; activation of the proteinase-activated receptor (PAR) family of G protein-coupled receptors (GPCR), with a mechanism in common with adhesion-triggered GPCRs (ADGRs); and by proteolytic ion channel regulation. These mechanisms are considered in the much wider context that proteolytic mechanisms serve, including the processing of growth factors and their receptors, the regulation of matrix-integrin signaling, and the generation and release of membrane-tethered receptor ligands. These signaling mechanisms are relevant for inflammatory, neurodegenerative, and cardiovascular diseases as well as for cancer. We propose that the inflammation-triggering proteinases and their proteolytically generated substrates represent attractive therapeutic targets and we discuss appropriate targeting strategies.

I. Introduction

Proteolytic enzymes or their inhibitors (e.g., the serpins) represent more than 2% of the human genome (Puente et al., 2005). Given that large genomic investment, it is not surprising that proteinases (colloquially termed “proteases”) are used physiologically to serve multiple biologic functions. Thus, added to their long-recognized roles as digestive enzymes for nutrient assimilation, proteinases can now be seen as “hormone-like” mediators that regulate target tissues by both nonreceptor and receptor-mediated mechanisms. Thus, in the setting of tissue inflammation triggered by injury or cancer, the activation and inactivation of proteinases in the extracellular microenvironment can regulate cell function in an autocrine-paracrine hormone-like manner as illustrated in Fig. 1. The central hypothesis that underlies this review is therefore that signaling by extracellular microenvironment proteinases caused by cleaving a number of distinct targets plays a major role in a wide spectrum of inflammatory diseases ranging from arthritis to colitis and cancer. These targets include the precursors that yield inflammatory peptides, cell membrane receptors including those for insulin and other growth factors, proteinase-activated G protein-coupled receptors (PARs) that play a central role in proteinase-stimulated signaling, integrin and adhesion receptors (ADGRs), and also ion channels (Fig. 2).

ABBREVIATIONS: ACE, angiotensin converting enzyme; ADGR, adhesion G protein-coupled receptors (formerly designated aGPCRs); APC, activated protein-C; ASIC, acid-sensitive ion channel; BACE, β-site of amyloid precursor protein; Der p, Dermatophagoides pteronyssinus; ENaC, epithelial sodium channel; EPCR, endothelial cell protein C receptor; GAIN, GPCR autoproteolysis-inducing domain of adhesion GPCRs; GPS, GPCR proteolysis site within the GAIN domain; KLK, kallikrein-related peptidase family that includes “prostate-specific antigen”/KLK3; MMP, matrix metalloproteinase; PAR, proteinase-activated receptor (PAR1, PAR2, PAR3, PAR4); PSA, prostate-specific antigen; TIMP, tissue inhibitor of metalloproteinase; TF, tissue factor; TL, tethered ligand that activates PARs upon proteolytic unmasking.
II. Classification and Overview of Mechanisms Regulating Proteolytic Enzymes

Proteolytic enzymes represent a diverse set of proteins, ranging in size from relatively small 20-kDa enzymes that fold to generate the catalytic pocket (e.g., cysteine cathepsins) to large multidomain complexes (e.g., the 26S proteasome ~2000 kDa). Based on their different catalytic mechanisms, proteinases are divided into five major classes named for the key amino acid (or metal ion) involved in peptide bond cleavage (Fig. 2). These classes include the serine, threonine, cysteine, aspartic, and metalloproteinases (see the MEROPS database: http://merops.sanger.ac.uk/). Of these, the serine, cysteine, and metalloproteinase families are large, with over a 100 members in each class, whereas the threonine and aspartic proteinase families are much smaller, with fewer than 30 members each (Lopez-Otin and Bond, 2008). Of relevance to the theme of proteinase-mediated signaling, each of the different proteinase classes can cleave the same target protein at distinct sites, and thus each enzyme can have distinct signaling properties although the target protein might be the same one. Given the chaos that unrestricted proteolysis could cause in biologic systems, proteinases are under strict regulatory control to ensure that their action occurs only in the appropriate context and only to a tightly limited extent. This issue is of particular importance in the setting of inflammation, where extensive proteolysis can occur. Thus endogenous proteinase inhibitors are as important for regulating signaling as are the proteinases themselves.

A number of mechanisms have evolved to regulate proteinase function. The first step in the regulation of proteinases occurs at the transcriptional level. Whereas proteasomal and lysosomal enzymes that are involved in general protein turnover are ubiquitously expressed, other proteinases exhibit significant restriction in their tissue and cellular distribution and in the timing of their expression. The selective expression of the...
neutrophil enzyme cathepsin-G in azurophilic granules (Salvesen et al., 1987; Conus and Simon, 2008) is an example of such selective transcriptional regulation of proteinase expression. In the context of inflammation, transcriptional regulation of both the proteinases and their inhibitors can also occur. For instance, a number of matrix metalloproteinases (MMPs) are reported to be upregulated during inflammation in a nuclear factor-κB-dependent manner (Bond et al., 1998, 2001; Trivedi et al., 2006). Furthermore, the inhibitors of the MMPs (tissue inhibitors of metalloproteinases, TIMPs) are also subject to change in inflammatory settings.

Another important mechanism by which proteinase function can be restricted to its site of action is through the synthesis of inactive zymogens, which require limited local proteolysis to be active on the final target substrate. The activation of zymogens can occur through autocatalysis, as is the case for a number of cysteine proteinases (Turk et al., 2001), or through the sequential “cascade” cleavage of a series of proteinase zymogens to generate active enzymes. This kind of proteolytic cascade, which results in a considerable amplification of the initial proteolytic signal, is best exemplified by the coagulation cascade that leads ultimately to the conversion of prothrombin to active thrombin (Davie and Ratnoff, 1964; Macfarlane, 1964). Both the complement and tissue kallikrein enzyme cascades generate comparable proteinase-triggered amplification networks (Yoon et al., 2007; Ricklin et al., 2010).

In addition to these regulatory mechanisms for activating zymogens and terminating proteinase action by autocatalysis or cleavage by other enzymes, enzyme activity is regulated further by endogenous enzyme-targeted inhibitors (Rawlings et al., 2004). It is interesting to note that relative to the large numbers of proteinases in the genome, the diversity of endogenous proteinase inhibitors, as outlined in Table 1, is more restricted (Potempa et al., 1994; Gomez et al., 1997; Gettins, 2002). For example only four endogenous metalloproteinase inhibitors, TIMP-1, TIMP-2, TIMP-3, and TIMP-4, have been identified for the large family of MMPs (Gomez et al., 1997; Clark et al., 2008; Khokha et al., 2013). Similarly, the numbers of cysteine proteinases exceed the numbers of their endogenous inhibitors, including the cystatins and thyropins (Turk et al., 2002; Dubin, 2005; Magister and Kos, 2013). Surprisingly, no endogenous inhibitors are known for the serine proteinase, tryptase, or the aspartic proteinases (Turk et al., 2012). The largest number of endogenous inhibitors is described for those that target serine proteinases, except for tryptase, designated by the acronym, SERPINS (SERine Proteinase INhibitorS) (Potempa et al., 1994; Law et al., 2006). In addition to these class-specific proteinase inhibitors, α2-macroglobulin is a circulating tetrameric plasma proteinase inhibitor that forms an enzyme-covalent complex with all four catalytic classes to inhibit a relatively large spectrum of different proteinases (Barrett and Starkey, 1973; Barrett, 1981; Rehman et al., 2013). Interestingly inhibition of an enzyme by α2-macroglobulin may block proteolysis of large substrates but may still allow cleavage of smaller substrates, such as in the case of KLK3-α2-macroglobulin or in the case of the alpha-2-macroglobulin-urokinase plasminogen activator complex, the latter of which has the ability to retain enzyme activity to cleave plasminogen, while at the same time being resistant to inhibition by the plasminogen activator inhibitor PAI-1 (Christensson et al., 1990; Komissarov et al., 2009). Of note, deficiencies in the actions of proteinase inhibitors, for example alpha-1-antitrypsin (also designated, alpha1-PI) or the Kazal type related inhibitor type 5, lead to a number of diseases like emphysema and skin inflammation (see Table 1 in Law et al., 2006; Hovnanian, 2013). Alternatively, the upregulation of SerpinA3N and its inhibition of leukocyte elastase in the dorsal root ganglion after nerve injury appear to be therapeutic in reducing neuropathic pain (Vicuña et al., 2015). That same study showed that the administration of this serpin can attenuate mechanical allodynia in a mouse model of pain. Thus both the downregulation and upregulation of proteinase inhibitors can play important physiologic roles in many settings.

To sum up, the inflammatory milieu can generate multiple proteinases of five catalytically distinct classes, along with a more restricted number of proteinase inhibitors (Table 1). These enzymes along with their

<table>
<thead>
<tr>
<th>Enzyme Family Inhibited</th>
<th>Inhibitor Class</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine proteinases</td>
<td>SERPINS (serine proteinase inhibitors)</td>
<td>Largest number of endogenous inhibitors; deficiencies cause lung and skin disease; upregulation involved in modulating neuropathic pain</td>
<td>Potempa et al., 1994; Law et al., 2006; Vicuña et al., 2015</td>
</tr>
<tr>
<td>Metalloproteinases (MMPs)</td>
<td>TIMPs 1, 2, 3, and 4</td>
<td>TIMPs elevated in cancer and inflammation; upregulation promotes fibrosis</td>
<td>Clark et al., 2008; Khokha et al., 2013; Arpino et al., 2015</td>
</tr>
<tr>
<td>Cysteine proteinases</td>
<td>Cystatins, thyropins</td>
<td>Intracellular inhibitors regulate apoptosis</td>
<td>Turk et al., 2002; Dubin, 2005; Magister and Kos, 2013</td>
</tr>
<tr>
<td>General</td>
<td>Alpha 2-macroglobulin</td>
<td>Alpha 2-M preserves catalytic site function for smaller substrates</td>
<td>Barrett and Starkey 1973; Barrett, 1981; Rehman et al., 2013</td>
</tr>
</tbody>
</table>

TABLE 1. Proteinase families and their endogenous inhibitors
inhibitors will differ from one inflammatory setting to another and thus their common and/or distinct substrate selectivities can influence the inflammatory signaling process in diverse ways. For that reason, proteinases have long been considered as important therapeutic targets for inflammatory disease. However, the complexities of their activation and inhibition, along with the common mechanisms of catalysis within a family have made the design of potent and enzyme-selective inhibitors very challenging. Nonetheless, a key issue to sort out is which proteinases and which molecular targets lead to signaling?

III. Proteinase-Mediated Signaling: Molecular Targets that Trigger the Response

A. Igniting the Inflammatory Response via Proteolytic Signaling

Amplified proteolytic signaling in a localized setting is of key importance in triggering an inflammatory response resulting from tissue injury or from invading infectious agents. Injury to a finger provides all of us with a familiar experience of this “hormonal role” of proteinases. Thus, shortly after the immediate “pain response” to the injury due to the stimulation of sensory nerves, the extrinsic and intrinsic coagulation pathways are activated, resulting in an amplified proteolytic signaling pathway that generates thrombin and other members of the coagulation cascade, including the counterregulatory proteinase, activated protein-C (APC). Shortly thereafter, 1) the blood clots, due to the proteolytic conversion of fibrinogen to fibrin and 2) all of the hallmark signs of inflammation are generated: more pain (dolor) and increased blood flow (rubor), resulting in increased warmth at the tissue surface (calor), increased fluid leakage with swelling (tumor), and subsequently, the scarring that can lead to reduced function (functio laesa).

This activation of the coagulation cascade is tightly linked to triggering of the complement and plasma kallikrein-kininogen systems, both of which represent enzymatic cascades that generate inflammatory peptides. After these responses generated by enzymes of the coagulation, kininogen, and complement cascades, the cellular “shock-troops” of inflammation (neutrophils) migrate into the site of injury to initiate a slightly delayed component of the acute inflammatory response. The influx of neutrophils, followed by other leukocytes, leads to proteinase secretion that stimulates further tissue responses along with the generation of cytokines in the microenvironment. All of these processes represent the “innate inflammatory response” aimed at combating the injurious stimulus and initiating the process of healing. All of these inflammatory responses are regulated by proteinase-mediated signaling, involving enzymes of the coagulation, kininogen, and complement cascades and proteinases secreted by the invading neutrophils and other cells at the site of injury.

B. Molecular Targets

The generic targets for proteinase-mediated signaling (Fig. 2) vary considerably in their protein sequences and anatomic-tissue specific locations. These substrates range from extracellular matrix components to very specific cell surface receptors like the one for insulin. Although the entire repertoire of proteinase-cleaved substrates is too large to cover in this review, we wish to highlight here the ability of proteinases to signal by generating extracellular bioactive peptides like the kinins and complement pathway-produced peptides, by activating cell surface receptors, including the insulin receptor and the PAR family of G protein-coupled receptors and by regulating ion channels. Together, these proteolytically triggered mechanisms control a wide variety of complex cell behaviors involved in the inflammatory response.


In addition to the well-recognized proteolytic generation of active peptide hormones from precursor polypeptides as alluded to above, e.g., via the action of prohormone convertases (Steiner et al., 1967; Steiner, 2011; Chretien, 2012), a number of proteolytic cascades are now known to yield inflammatory peptides by processing extracellular rather than intracellular substrates, via mechanisms outlined elsewhere in some detail (Turk et al., 2012).

The coagulation cascade (Fig. 3), which triggers cell signaling via the G protein-coupled proteolytically activated receptor family (PARs), is also linked to two other plasma-localized proteolytic cascades that generate inflammatory polypeptide signals: the complement system (Fig. 4) and the plasma kallikrein-kininogen-kinin system (Fig. 5). Another more recently recognized cascade, that of the tissue kallikrein family, now termed kallikrein-related peptidases (KLKs), is also coming into sharper focus as a multiproteinase inflammation-related cascade network that can be activated in settings of inflammatory disease (Yoon et al., 2007; Sotiropoulou and Pampalakis, 2010; Hovnanian, 2013; de Veer et al., 2014). These interconnected proteolytic “signal amplification” cascades represent key constituents of the innate immune inflammatory responses to injury, and their signaling is coordinated with that of the coagulation cascade (Joseph and Kaplan, 2005; Kaplan and Ghebrehiwet, 2010).

2. Complement and Other Interconnected Proteolytic Cascades.

The complement proteolytic cascade is well-recognized as a source of inflammatory peptides (Fig. 4). These peptides signal to cells via their peptide-selective G protein-coupled receptors (Marceau and Regoli, 2004; Leeb-Lundberg et al., 2005; Alexander et al., 2013). What is now evident is that not only is the complement cascade linked to the coagulation cascade (Fig. 3, top...
Proteinase-Mediated Signaling and Inflammation

Both the KLKs and the coagulation enzymes can cause the generation of the complement-derived molecules, C3a, C5b, C5a, or C5b, which among other actions can also, like thrombin, regulate platelet function [aggregation; ADP release (Polley and Nachman, 1983)] and trigger tissue inflammation via their G protein-coupled receptor targets, C3AR1 and C5AR1 (Alexander et al., 2013; Klos et al., 2013). This generation of complement-derived inflammatory peptides can thus occur in step with the ability of the coagulation cascade enzymes, the plasma kallikrein, and the KLKs to liberate kinins and to activate PARs. It is thus possible to link the activation of target cells by enzymes of the coagulation pathway with those triggered by the complement system along with the kininogen and KLK cascade systems. A common theme for these proteolytic systems is that they represent an amplification mechanism for sensing injury because of 1) the large signal magnification process inherent in a catalytic proteolytic cascade and 2) the subsequent activation of “hormone-like” receptor-mediated signaling pathways that in turn greatly amplify the initial proteinase-triggered signal.

The complement network that generates the inflammatory peptides consists of more than 50 plasma-borne and membrane-bound proteins that form an essential component of the innate defense system. This network forms a first line of defense against microbial invaders and exerts important functions in immune surveillance and homeostasis (Ricklin et al., 2010). Activation of the complement system is primarily initiated by pathogen- and damage-associated molecular surface patterns (often abbreviated by the acronyms PAMPs or DAMPs) acting via cell surface receptors. The complement-generated process is commonly categorized under three major pathways (classic, lectin, and alternative) of stepwise proteolytic activation (Fig. 4). However, there is increasing evidence for direct activation of individual complement components by proteinases extrinsic to the complement cascade, as part of an intricate crosstalk between physiologic effector systems (Le Friec and Kemper, 2009; Ricklin et al., 2010). Under this extrinsic influence, active complement factors C3a and C5a (also known as anaphylatoxins) and inactive derivatives are known to be released by the proteolytic action of trypsin (Wetsel and Kolb, 1982; Eggertsen et al., 1985), plasma...
Plasma kallikrein, or the Kallikrein-kinin System. The recent availability of C3AR1-targeted agonist ligands will enable a more extensive interrogation of this area of interest (Reid et al., 2014). Not surprisingly, the downstream signaling initiated by the C3AR1 receptors (Klos et al., 2009, 2013; Alexander et al., 2013). Surprisingly, only one of the KLK family members, namely KLK1 (termed tissue kallikrein in the older literature) is also able to generate active kinins from a kininogen precursor.

4. Kallikrein-Related Peptidases and the Production of Inflammation-Related Peptides. The KLKs, belong to an extensive family of tissue serine proteinases with trypsin or chymotrypsin-like activity. Biochemically the KLKs are distinct from plasma kallikrein both in structure and substrate specificity (Borgono and Diamandis, 2004; Sotiropoulou and Pampalakis, 2010). The best known member of this family, now termed KLK3, was previously known as an androgen-regulated prostate-specific antigen (PSA). PSA was first discovered in 1979 as a unique antigen in human prostatic tissue extracts (Wang et al., 1979, 1981). Although this antigenic property of PSA was rapidly recognized, the proteolytic activity of human PSA, like that of the comparable KLK serine proteinase in canine seminal plasma, was not appreciated for another 5 years (Ban et al., 1984; Isaacs and Coffey, 1984). PSA/KLK3 is well-recognized as a biomarker for prostate cancer monitoring (Stephan et al., 2007; Bryant and Lilja, 2014). It is now known that this enzyme belongs to a large family of serine proteinases, which are encoded on chromosome 19 of the human genome, consisting of 15 distinct enzymes [KLK1 to KLK15 (Borgono and Diamandis, 2004; Sotiropoulou and Pampalakis, 2010)]. KLK3/PSA, along with KLKs 7 and 9 are “chymotryptic” in terms of their substrate specificity, whereas the other 12 members of the human KLK family have a trypsin-like selectivity (Clements et al., 2001; Yousef and Bhoola et al., 1992). The plasma kallikrein enzyme that generates the kinins has a number of functional protein domains in addition to its catalytic domain responsible for substrate cleavage (Chung et al., 1986; Bhoola et al., 1992). The plasma mechanism by which high molecular weight kininogen is processed to bradykinin and other inflammatory peptides is linked tightly to the proteinase cascades for coagulation and fibrinolysis (Joseph and Kaplan, 2005). Thus coagulation Factor XIIa is involved not only in the downstream conversion of prothrombin to thrombin, but also in the conversion of prekallikrein to plasma kallikrein. As a major function, plasma kallikrein, a key player in the innate inflammatory response, catalyses the release of the inflammatory peptide bradykinin from a high molecular weight precursor molecule (high molecular weight kininogen) produced by the liver. In turn, bradykinin triggers signals via the bradykinin G protein-coupled receptors (mainly B2, also possibly B1). There is a longstanding extensive literature to which the reader is referred (Bhoola et al., 1992; Marceau and Regoli, 2004; Leeb-Lundberg et al., 2005; Moreau et al., 2005; Whalley et al., 2012) dealing both with the biochemistry of the kinin-forming proteolytic cascade and with agonists and antagonists for the target bradykinin B1 and B2 receptors that convey the inflammatory signals.

Given the involvement of this proteolytic system in disorders of pain and inflammation, there are a number of therapeutic settings in which plasma kallikrein, the kinins, and their receptors are of importance (Marceau and Regoli, 2004; Joseph and Kaplan, 2005; Moreau et al., 2005). The KLKs, belong to an extensive family of tissue serine proteinases with trypsin or chymotrypsin-like activity. Biochemically the KLKs are distinct from plasma kallikrein both in structure and substrate specificity (Borgono and Diamandis, 2004; Sotiropoulou and Pampalakis, 2010). The best known member of this family, now termed KLK3, was previously known as an androgen-regulated prostate-specific antigen (PSA). PSA was first discovered in 1979 as a unique antigen in human prostatic tissue extracts (Wang et al., 1979, 1981). Although this antigenic property of PSA was rapidly recognized, the proteolytic activity of human PSA, like that of the comparable KLK serine proteinase in canine seminal plasma, was not appreciated for another 5 years (Ban et al., 1984; Isaacs and Coffey, 1984). PSA/KLK3 is well-recognized as a biomarker for prostate cancer monitoring (Stephan et al., 2007; Bryant and Lilja, 2014). It is now known that this enzyme belongs to a large family of serine proteinases, which are encoded on chromosome 19 of the human genome, consisting of 15 distinct enzymes [KLK1 to KLK15 (Borgono and Diamandis, 2004; Sotiropoulou and Pampalakis, 2010)]. KLK3/PSA, along with KLKs 7 and 9 are “chymotryptic” in terms of their substrate specificity, whereas the other 12 members of the human KLK family have a trypsin-like selectivity (Clements et al., 2001; Yousef and
Diamandis, 2001). These enzymes, well-recognized as fruitful cancer biomarkers, are now known to be widely expressed in many organs and to have a broad impact on cell function (Kuriyama et al., 1980; Yousef and Diamandis, 2001, 2009; Sotiropoulou et al., 2009). In rodents and other species, gene duplication has resulted in the generation of more than 25 KLK genes, some of which are pseudogenes and are not expressed as functional proteinases (Clements, 2008; Lundwall and Brattsand, 2008; Lundwall, 2013). The KLKs are upregulated hormonally (e.g., androgens, estrogens, progestins, vitamin D) in many tissues, mainly of epithelial origin (Lawrence et al., 2010), and can be found in tumor-derived biologic fluids, such as ascites fluid and in sera from cancer patients. A wide array of potential substrates has been identified whereby KLKs may affect cell function (Sotiropoulou et al., 2009; Lawrence et al., 2010). In this context, studies done in vitro have singled out proteins of the extracellular matrix, pro-uromodulin-plasminogen activator (pro-uPA), kininogens, growth factor precursors (and binding proteins), cytokines, insulin-A and -B chains, and other KLKs, all as potential targets of kallikrein proteolysis, particularly in the setting of cancer progression (Frenette et al., 1997; Takayama et al., 2001; Borgono and Diamandis, 2004; Lawrence et al., 2010). A recent “degradomic” approach has identified other potential KLK substrates cleaved in cultured ovarian cancer cells by the combined actions of KLKs 4, 5, 6, and 7 (Shahinian et al., 2014). Such substrates (e.g., extracellular matrix; pro-uromodulin-plasminogen activator, growth factor precursors, kininogens, TGFβ-1-precur- sor) may well explain some but by no means all of the physiologic signaling actions of the KLKs, particularly in the setting of inflammatory disease and cancer. The precise mechanisms whereby the KLKs can play an active role in pathophysiology are not fully understood. Some concrete examples of KLK-dependent signaling are discussed in the following sections.

5. Generation of Kinins by Kallikrein-Related Peptidase 1

A concrete example of KLK-dependent signaling can be seen for KLK1. Like plasma kallikrein (above), one of the KLK family members, namely KLK1, can produce bradykinin receptor-stimulating peptides from a kininogen precursor. Thus KLK1, also formerly known as tissue, urinary, pancreatic, or renal kallikrein, unlike the other KLKs, possesses kininogenase activity. This activity is responsible for the cleavage of a low molecular weight precursor kininogen, produced by the liver, to release lysyl-bradykinin (Fig. 5) (Moreau et al., 2005). Lysyl-bradykinin in turn activates the bradykinin B1 receptor (Leeb-Lundberg et al., 2005; Moreau et al., 2005). KLK1, via its production of lysyl-bradykinin from kininogen has been implicated in various physiologic processes, including the control of blood pressure and electrolyte balance, as well as in inflammation (Clements, 1989; Margoliou, 1998a,b).

6. Interplay between Kallikrein-Related Peptidases, Coagulation Proteinases, and the Complement Cascade.

The coagulation cascade is integrally linked to the complement and kallikrein-kinin cascades. Observations in the 1980s pointed to the existence of significantly higher levels of complement activation products in human serum compared with anticoagulated blood, strongly suggesting the development of complement activation during blood clotting (Mollnes et al., 1988). It is now clear that there is crosstalk between the complement and coagulation cascades, either at the level of anaphylatoxin generation (Wiggins et al., 1981; DiScipio, 1982; Hiemstra, 2002; Amara et al., 2010), upstream in the complement pathways (Ghebrehiwet et al., 1981, 1983), or via the generation of activated thrombin (Krarup et al., 2007; Gulla et al., 2010). Complement effectors can also facilitate biochemical and morphologic changes that can indirectly affect coagulation (Oikonomopoulou et al., 2012). For example, the anaphylatoxin C3a and its derivative C3a desArg can induce platelet activation and aggregation directly (Polley and Nachman, 1983).

The KLKs are also known to interconnect with the coagulation proteinases, mainly by cross-activating/deactivating proteinases of each other’s cascade or by affecting receptor signaling, e.g., via the proteinase-activated receptor family (PARs) and the urinary plasminogen activator receptor uPAR (Blaber et al., 2010). Furthermore, an impact of the KLKs on the complement cascade is also now known (Oikonomopoulou et al., 2013). Thus KLKs are able to generate the anaphylatoxin, C3a peptide, by acting on C3 outside of the “regular” complement activation cascade. In the case of KLK14, the anaphylatoxin was shown to possess significant biologic functions (Oikonomopoulou et al., 2013). In summary, four key proteolytic cascades, involving enzymes of the coagulation pathway, the complement system, plasma kallikrein, and the kallikrein-related peptidase/KLK family play important roles in the innate inflammatory response by generating active peptides that signal to cells via their specific receptors. This “indirect mode” of regulating cell signaling via the generation of peptide agonists for receptors is paralleled by the direct action of some of these enzymes to regulate a number of cell surface receptors, including growth factor receptors like the one for insulin, G protein-activated receptors, and ion channels, as outlined in the following sections.

C. Hormone-like Signaling by Proteinases: Mimicking the Action of Insulin and Mitogenic Polypeptides

In the mid-1960s, it was discovered that enzymes like trypsin and chymotrypsin can cause hormone-like metabolic-anabolic effects in tissues and cells akin to the actions of insulin (Rieser and Rieser, 1964; Rieser, 1967; Kono and Barham, 1971). This “insulin-like” action of trypsin on fat cells and rat diaphragm tissue
is now known to result from the cleavage of the extracellular alpha-subunit of the insulin receptor. That cleavage removes a negative regulatory domain from the extracellular insulin receptor alpha-subunit to release its inhibitory control of receptor function and to enable receptor autophosphorylation-activation (Shoelson et al., 1988). At higher trypsin concentrations, the insulin binding site on the receptor is removed, thus “disarming” the receptor and preventing insulin-triggered signal transduction (Cuatrecasas, 1969, 1971). These data obtained for signaling by the insulin receptor very likely also apply to signaling by the insulin receptor very likely also apply to signaling by the insulin-like growth factor receptors. The data obtained for the effect of trypsin on insulin signaling established the principle that tissue proteinases released at sites of inflammation can have a “bidirectional” direct impact on receptors both to activate and silence signaling. These anabolic actions of trypsin and other proteinases mimic the mitogenic receptor-mediated effects of polypeptide growth factors such as insulin and epidermal growth factor (Burger, 1970; Sefton and Rubin, 1970; Chen and Buchanan, 1975; Carney and Cunningham, 1977, 1978). To date, this effect of tissue proteinases on insulin receptor signaling, and by extension, signaling via the insulin-like growth factor-1 receptor, has gone largely unrecognized and has not yet been explored in any depth. In principle, this proteolytic mode of activation of the insulin receptor could play a role in inflammatory disease (Hyun et al., 2010). The impact of proteinases on the regulation of other “growth factor” receptors is a topic of interest for further study. The action of trypsin to signal via the insulin receptor heralded the discovery of its ability to regulate cell function by activating a novel family of G protein-coupled receptors, the “PARs.” The theme that links the proteinase-mediated activation of a growth factor receptor and a G protein-coupled receptor is that there is a commonality in the downstream signal pathways that are ultimately triggered by both kinds of receptors.

D. Proteinase-Activated G Protein-Coupled Receptors: A New Paradigm for G Protein-Coupled Receptor Signaling

At the time the Riesers discovered the anabolic insulin-like actions of trypsin and other proteinases in striated muscle (Rieser and Rieser, 1964; Rieser, 1967), it also became clear that the catalytic activity of thrombin and other serine proteinases can regulate platelet function (Davey and Luscher, 1967; Ganguly, 1974; Martin et al., 1975). However, the mechanisms whereby the proteolytic enzymes activated platelets and stimulated fibroblast mitogenesis were not known. It was not until the early 1990s that a receptor mechanism for these “hormone-like” actions of proteolytic enzymes was discovered. That discovery resulted from a search that spanned over 30 years (time frame shown in Table 2) to identify the “receptors” responsible for the action of thrombin 1) to stimulate human platelet function and 2) to trigger mitogenesis in cultured cells. To the surprise of many, it turned out that thrombin causes these effects by stimulating a proteolytically activated receptor (PAR) that is a member of the G protein-coupled receptor (GPCR) superfamily (Rasmussen et al., 1991; Vu et al., 1991; Adams et al., 2011). This receptor mechanism whereby proteinases can signal to cells is shown in Fig. 6. One main focus of this review is on these proteolytically triggered proteinase-activated receptors or PARs, for which the unique mechanism of proteolytic activation was discovered by the Coughlin laboratory (Vu et al., 1991). It is important to recognize that although thrombin was the index proteinase leading to the discovery of the PARs, enzymes other than those of the coagulation cascade, like trypsin, the KLKs, and neutrophil-secreted enzymes can also signal via these unique receptors. Furthermore, after the discovery of PAR1 (known only as “the thrombin receptor” at the time), additional pharmacological (Hollenberg et al., 1993; Kinlough-Rathbone et al., 1993) and gene knockout studies very rapidly pointed to the existence of additional members of this proteolytically activated family of receptors. As outlined in Tables 2 and 3, continued work then led to the discovery of PAR2 (Nystedt et al., 1994; Bohm et al., 1996b), PAR3 (Ishihara et al., 1997), and finally PAR4 (Kahn et al., 1998; Xu et al., 1998). The PAR-triggered signaling mechanism, representing a paradigm shift for understanding proteinase function, can be seen to be complementary to the other mechanisms illustrated in Fig. 2, whereby circulating and tissue-derived proteinases can play a role in inflammation.

1. “Tethered Ligand” Receptor Domains Responsible for Proteinase-Activated Receptor Activation. In addition to cloning the human PAR1 thrombin receptor, the Coughlin group showed that the mechanism whereby thrombin activates PAR1 involves the proteolytic unmasking of a cryptic N-terminal receptor ligand, the so called tethered ligand (TL), that remaining attached, activates the receptor as shown in Fig. 6A, middle. The mechanism identified for PAR1 holds true for PAR2 and PAR4 as well. PAR2 is preferentially activated by trypsin, although at relatively high concentrations thrombin can also activate PAR2 (Mihara et al., 2016). Moreover, both trypsin and thrombin can activate PAR4 with comparable potencies. The revealed tethered ligand of the PARs is thought to interact with extracellular loop-2 of the receptor (Lerner et al., 1996; Al-Ani et al., 1999). In the case of PAR3, thrombin cleavage can also expose a tethered ligand sequence, but the ability of this sequence to cause PAR3 activation to signal on its own is still unclear, despite some evidence in favor of independent signaling due to PAR3 activation (Ostrowska and Reiser, 2008). Instead, PAR3 appears to act as a cofactor for thrombin-mediated activation of
PAR4 (Nakanishi-Matsui et al., 2000). PAR3 may also signal as part of a heterodimer with PAR1, wherein the unmasked TL of PAR3 “reaches over” to activate PAR1 (McLaughlin et al., 2007). Part of the confusion about the signaling role of PAR3 stems from the fact that synthetic peptides based on its tethered ligand sequence unmasked by thrombin efficiently activate PARs 1 and 2 (Hansen et al., 2004). Thus, some of the effects attributed to PAR3 due to the actions of the PAR3-derived tethered ligand peptides are very likely due to PARs 1 and 2 and not to PAR3 as suggested by some publications.

2. Synthetic Receptor-activating Peptides Based on the Tethered Ligands. Along with the discovery that the thrombin PAR1 receptor is activated by a cryptic tethered ligand peptide sequence, Coughlin and colleagues found that short synthetic peptides based on the tethered ligand sequence could also trigger activation of PAR1 without the need for receptor proteolysis (Fig. 6B, right) (Vu et al., 1991). In a similar way, synthetic peptides based on the cryptic tethered ligand sequences unmasked by proteolysis in PARs 2 and 4 have been developed for the selective activation of PARs 2 and 4 (Ramachandran et al., 2012b). Although these PAR-activating peptides are quite selective for activating the PARs (e.g., TFLLR-amide for PAR1, 2-furoyl-LIGRLO-amide for PAR2; AYPGKF-amide for PAR4), there can be some off-target actions of the peptides. For instance, the PAR2-activating peptides can cause effects in PAR2-null mice (McGuire et al., 2002), possibly by activating the Mas-related G protein-coupled receptor, MrgprC11 (Liu et al., 2011). These off-target effects of the PAR-activating peptides can be resolved by obtaining structure-activity profiles of the peptide-generated responses (Liu et al., 2011).

3. “Noncanonical” Proteinase-Activated Receptor Tethered Ligands Revealed by Proteinases other than Thrombin and Trypsin. In the studies resulting in the cloning of PARs 1, 2, and 4, the key cleavage site for both thrombin (PARs 1 and 4) and trypsin (PAR2) (here designated by: //) was found to be at the serine proteinase-targeted N-terminal arginine in the human PARs: R//SFLLRN— for PAR1; R//SLIGKV— for PAR2; and R//GYPGQV— for PAR4. However, it subsequently became apparent that, like trypsin and thrombin, other serine proteinases (for instance, the KLK peptidases), could also unmask the same tethered ligands revealed by thrombin and trypsin. Nonetheless, it was soon found that quite different proteinases [e.g., matrix metalloproteinase-1 (MMP-1), activated protein-C (APC), neutrophil elastase, proteinase-3, and cathepsin-S] could also cause PAR activation/signaling by cleaving at distinct N-terminal residues to unmask different “noncanonical” receptor-activating tethered ligand’ sequences (Boire et al., 2005; Ramachandran et al., 2011; Mosnier et al., 2012; Mihara et al., 2013;
Schuepbach et al., 2012; Zhao et al., 2014a,b). Unmasking of these distinct noncanonical tethered ligand sequences triggers so-called functional selectivity or biased PAR signaling (Kenakin, 2011, 2013; Kenakin and Miller, 2010; Kenakin and Christopoulos, 2013) that specifically activates unique downstream signaling pathways that differ from those triggered by either thrombin (for PAR1) or trypsin (for PAR2). Moreover, synthetic peptides based on these noncanonical tethered ligand sequences can also behave as biased agonists for the PARs (Mihara et al., 2013). The multiple sites at which PARs 1 and 2 can be cleaved to unmask either nonbiased or biased tethered activating ligands is illustrated by the sequences at Fig. 6, bottom. It is also possible that proteinase cleavage of the extracellular domains or a PAR can destabilize receptor conformation to stimulate effector interactions and signaling via a process that does not involve docking of a PAR tethered ligand. This diversity of tethered ligands that are sequestered in the N-terminal sequences of the PARs and the possible activation of PARs via a tethered ligand-independent mechanism leads to a substantial flexibility with which microenvironment inflammation-related proteinases can regulate signaling via the PARs. Thus in principle, three outcomes are possible for the impact of proteinases on PAR signaling: 1) canonical unbiased signaling via cleavage at the serine proteinase sites singled out by thrombin or trypsin; 2) biased signaling, triggered by cleavage at the noncanonical sites targeted by matrix metalloproteinases-1 and -13 (MMP1; MMP13), activated protein-C (APC), neutrophil proteinases (elastase and proteinase-3), cathepsin-S, and by the other enzymes shown in Fig. 6, bottom; 3) silencing of signaling caused by cleavage at site(s) that remove any potential tethered ligand sequences to prevent signaling (i.e., disarming by removing the thrombin-unmasked tethered ligand sequence shown in Fig. 6A). This disarming can be caused by the kallikrein-related peptidases, KLKs 8 and 14 (Oikonomopoulou et al., 2006; Ramachandran et al., 2012a). As summarized in Table 1, substantial progress has been made in identifying proteinases that can regulate the PARs and in developing agonists and antagonists for PARs 1, 2, and 4.

4. Regulation of Proteinase-Activated Receptor Signaling: Desensitization, Internalization, and Intracellular Targeting. Once unmasked by a PAR-activating proteinase, the tethered ligand remains attached to the

---

**Fig. 6.** Signaling by proteinase-activated receptors. (A) Proteinase-activated receptors are a family of GPCRs activated by proteolytic processing of the receptor N terminus to unmask a cryptic receptor-activating tethered ligand. The middle panel (A) illustrates this activation process, with the proteolytically revealed tethered ligand (SFLLRN: gray sequence) shown as nestled into the receptor. The receptor can also be “disarmed” by proteinases that cleave downstream of the tethered ligand sequences thereby preventing receptor activation by “canonical” activators like thrombin (PARs 1 and 4) or trypsin (PAR2). (B) Receptor activation can be triggered in the absence of proteolysis by the exogenous addition of synthetic peptides that mimic the proteolytically revealed ligand (TFLLR: pink sequence). As outlined in the text, cleavage at PAR1 sites other than the one targeted by thrombin can also cause receptor activation by unmasking a noncanonical tethered ligand that generates biased signaling. The possible activation of PAR signaling via a tethered ligand-independent process is not shown. (C) The lower panel shows the sequence of PAR1 and its sites of cleavage by enzymes that stimulate either biased or unbiased signaling. This tethered-ligand mechanism resulting from receptor proteolysis is in common for all of the PARs. APC, activated protein C; CG, cathepsin-G; MMP-1,13, matrix metalloproteinases 1 and 13; NE, neutrophil elastase; PR3, proteinase-3; VIIa/Xa, coagulation factor VIIa/Xa complex.
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Gene</th>
<th>Agonist Proteinases</th>
<th>Agonist Peptide</th>
<th>Peptido-Mimetic Agonist</th>
<th>Antagonist or Inhibiting Proteinases</th>
<th>Radioligand</th>
<th>G Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR1</td>
<td>F2R (Hs), F2r (Mm), F2r (Rn)</td>
<td>Thrombin (Vu et al., 1991)</td>
<td>TFLLR-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>None</td>
<td>Vorapaxar (Checckalamannil et al., 2005, 2008), Atopaxar (Kogushi et al., 2011), RWJ56110 (Andrade-Gordon et al., 1999) KKL4 possible disarming (Ramsay et al., 2008) KKL5 disarming (Ramachandran et al., 2012a) KKL4 disarming (Oikonomopoulou et al., 2006a,b)</td>
<td>[3H]haTRAP (Ahn et al., 1997)</td>
<td>Gq/11, Gi/o, G12/13</td>
</tr>
<tr>
<td>PAR2</td>
<td>F2RL1 (Hs), F2rl1 (Mm), F2rl1 (Rn)</td>
<td>Trypsin (Nystedt et al., 1994, 1995a,b; Bohn et al., 1996a; Saiededdine et al., 1996)</td>
<td>2f-LIGRL-O-NH&lt;sub&gt;2&lt;/sub&gt; (McGuire et al., 2004), SLIGRL-NH&lt;sub&gt;2&lt;/sub&gt;, SLIGKV-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>GB110 (Barry et al., 2010), AC-55541 and AC-264613; (Gardell et al., 2008)</td>
<td>GB88 (Suen et al., 2012); P&lt;sub&gt;2&lt;/sub&gt;pal18S pepducin (Sevigny et al., 2011)</td>
<td>[3H]propionyl-O-NH&lt;sub&gt;2&lt;/sub&gt; (Hollenberg et al., 2008), [3H]2-furoyl-LIGRL-NH&lt;sub&gt;2&lt;/sub&gt; (Banke et al., 2005)</td>
<td>Gq/11, Gi/o, G12/13</td>
</tr>
</tbody>
</table>
receptor and is thus not able to diffuse away, as is the case for conventional G protein-coupled receptor agonists. Thus the mechanisms that regulate PAR signaling would be expected to be in many respects distinct from those that affect other agonist-activated GPCRs, e.g., in terms of internalization and recycling to the cell surface after activation. A number of studies have been done to understand this aspect of the molecular machinery involved in the trafficking of PAR1 and PAR2. However, in contrast to PARs 1 and 2, limited insights are available for understanding the dynamics of PAR4 signaling and the potential role(s) that PAR3 mobility and internalization might play in terms of signal transduction (Marchese et al., 2008; Adams et al., 2011). What is clear is that the control of PAR signaling occurs on several levels, in terms of both 1) the time frame of signaling desensitization and 2) the process of receptor internalization, turnover, and trafficking of new receptors to the cell surface. These mechanisms are discussed here because they may well be subject to regulation by extracellular proteinases such as chymotrypsin, which has been observed to target a cell surface event subsequent to platelet activation by thrombin, but before the PAR-stimulated platelet response (Tam et al., 1980). Of note, PAR1 is reported to internalize through both constitutive and agonist-triggered internalization pathways (Shapiro et al., 1996; Shapiro and Coughlin, 1998). This process of constitutive internalization has not yet been evaluated in depth for either PAR2 or the other PARs. Whether extracellular membrane constituents involved in receptor trafficking and internalization after PAR activation might be regulated by proteolysis is an issue that merits attention.

5. Proteinase-Activated Receptor 1. PAR1 internalization is dependent on dynamin and clathrin-coated pits and involves an activation-dependent phosphorylation of the receptor via the receptor-targeted kinases GRK3 and GRK5 (Ishii et al., 1994; Hammes et al., 1999; Trejo et al., 2000). The requirement for arrestins for the internalization of PAR1 is unclear (Chen et al., 2004). It is suggested that PAR1 can interact directly with the clathrin adaptor AP2 complex through the YXXL motif (Paing et al., 2004; Dores et al., 2012). A second tyrosine motif proximal to the transmembrane domain is also present in the PAR1 C terminus and interacts with AP3 (Canto and Trejo, 2013). Constitutive internalization of unactivated PAR1 is AP2 dependent and negatively regulated by ubiquitination (Wolfe et al., 2007; Chen et al., 2011), whereas the trafficking of internalized receptor from endosomes to lysosomes is AP3 dependent (Canto and Trejo, 2013). After activation, basally ubiquitinated PAR1 is deubiquitinated by unknown mechanisms and is then phosphorylated and internalized in a bicaudal D1-dependent mechanism to early endosomes (Swift et al., 2010). Activated PAR1 is then sorted through late endosomes and targeted to lysosomes for degradation in a process dependent

### TABLE 3—Continued

<table>
<thead>
<tr>
<th>Receptor</th>
<th>G Protein</th>
<th>Antagonist or Inhibiting Proteinases</th>
<th>Antagonist Peptide</th>
<th>Agonist Proteinases</th>
<th>Peptido-Mimetic Agonist Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR3</td>
<td>Gq/11, Gαi</td>
<td>None</td>
<td>None</td>
<td>KLK14 (Oikonomopoulou et al., 2006a,b; Stefansson et al., 2008; Gratio et al., 2011; Chung et al., 2012; Ramachandran et al., 2012a)</td>
<td>None</td>
</tr>
<tr>
<td>PAR4</td>
<td>Gq/11, Gi/o</td>
<td>Trypsins (Xu et al., 1998)</td>
<td>AYPGKF-NH2 (Faruqi et al., 2000; Hollenberg and Saifeddine, 2001)</td>
<td>Thrombin (Kahn et al., 1998; Xu et al., 1998)</td>
<td>YD-3 (Wen et al., 2002); ML754 (Young et al., 2014); ML885 (Wen et al., 2014)</td>
</tr>
</tbody>
</table>
PAR2 is phosphorylated and binds (Bohm et al., 1996a). In contrast with PAR1, activated Par izes solely through agonist-triggered mechanisms and for activation triggered by nonbiased PAR1-activating peptides, the situation for the acti-

vation of the receptor via a noncanonical tethered ligand or via a biased peptide agonist has yet to be evaluated. Thus neutrophil-elastase-activated PAR1 remains at the cell membrane (Mihara et al., 2013), and the mechanisms of its activation-dependent desensitization and turnover remain a most interesting topic for further study.

6. Proteinase-Activated Receptor 2. PAR2 internal-
izes solely through agonist-triggered mechanisms (Bohm et al., 1996a). In contrast with PAR1, activated PAR2 is phosphorylated and binds β-arrestin 1 and β-arrestin 2 (Dery et al., 1999). PAR2 internalizes to the early endosomes in an ubiquitination-dependent process also requiring the GTPase Rab5a (Roosterman et al., 2003; Jacob et al., 2005). Continued sorting of PAR2 requires hepatocyte growth factor-regulated tyrosine kinase substrate (Hasdemir et al., 2007). In the late endosomes, PAR2 is deubiquitinated before lysosomal degradation. PAR2 resensitization at the cell surface with de novo Golgi synthesized receptors occurs in a Rab11a-dependent process (Roosterman et al., 2003). As for the noncanonical activation of PAR1 via enzymes like neutrophil elastase, the activation of PAR2 via neutrophil elastase does not trigger either PAR2 internalization or the activation of Gq-coupled calcium signaling (Ramachandran et al., 2009, 2011). Thus, for both PARs 1 and 2, the dynamics and location of receptor activation (i.e., cell surface versus internal-
ized scaffolds) plays a key role in the activation of distinct downstream signaling pathways.

7. Proteinase-Activated Receptors 3 and 4. Mechanisms contributing to PAR3 and PAR4 internal-
ization and trafficking remain largely unknown. In the case of PAR4, it is reported that the kinetics of signal termination after agonist occupancy is slower compared with PAR1 and PAR2 (Shapiro et al., 2000); however, the underlying mechanisms are unknown. Further-
more, the impact of proteolytic activation of PAR4 via a noncanonical tethered ligand on receptor trafficking has yet to be evaluated.

8. Effectors that Mediate Proteinase-Activated Receptor Signaling. In keeping with the “mobile” or “floating” model of receptor function (de Haen, 1976; Jacobs and Cuatrecasas, 1976), it is not surprising that PARs, like other receptors, can regulate multiple effec-
tors to result in biased signaling via processes described in detail by Kenakin and coworkers (Kenakin and Miller, 2010; Kenakin, 2013; Kenakin and Christopou-
os, 2013; Christopoulos et al., 2014). Of importance, we also know now that, like other GPCRs, the PARs can signal as either homo- or heterodimers as reviewed elsewhere (Gieseler et al., 2013). However, this area is not sufficiently clarified for its impact on signaling and will not be dealt with here. Nonetheless, either as a monomer or dimer, PAR2 can signal via multiple effectors.

Although G protein interactions are without doubt a key for PAR signal transduction (e.g., Gq, Gi, G12/13), PAR signaling can in principle involve a variety of other signal effectors. For instance, PAR2 signaling that does not depend on Gq-alpha is driven by an internalized PAR2-beta-arrestin scaffold that leads to MAPKinase signaling (Defea, 2008). Furthermore, the interactions of PARs with selected G proteins can involve multiple additional effectors. For instance, the diseveled pro-
tein, an upstream Wnt signaling protein, can be selec-
tively bound to the PAR1-activated G13 alpha-subunit to affect beta-catenin signaling (Turm et al., 2010). The C-terminal domains of the PARs are also involved in such receptor-effector interactions, possibly involving PAR-PAR homo- or heterodimer-effector interactions as alluded to above (Gieseler et al., 2013). In particular, C-terminal sequences in PAR2 have been found to regulate calcium and MAPKinase signaling and to modulate Akt activation via a plekstrin homology-domain binding motif (Seatter et al., 2004; Kancharla et al., 2015).

In summary, the PARs are able to generate signals by interacting with multiple effectors that, depending on the cell host, may drive quite distinct signals. The differential signaling triggered by PARs either via distinct effector interactions in different cell environ-
ments or via biased signaling stimulated by signal-
selective enzymes or synthetic agonists remains a fruitful area for further study.

9. Posttranslational Modifications of Proteinase-
Activated Receptors and Proteinase-Activated Receptor Function. Soon after the cloning of PAR1 it was recognized that the PARs could be regulated by post-
translational modifications. Using an antibody that targeted the N terminus of PAR1, Brass et al. (1992) showed that PAR1 migrated as a 66-kDa protein on Western blots, as opposed to the mass of the receptor predicted from its amino acid sequence of around 35 kDa. Subsequent work has shown that PAR1 glycosylation is important for plasma membrane expression of the receptor (Xiao et al., 2011) as well as for ligand-induced receptor activation and internalization (Soto and Trejo, 2010). Similarly, our own work showed that PAR2 is also extensively glycosylated and that loss of glycosylation sequons negatively affects cell surface receptor expression (Compton et al., 2002). In the case of PAR2, it was demonstrated further that glycosyla-
tion of the receptor also serves to regulate activation of the receptor by endogenous enzymes, with mast cell-derived tryptase being unable to cleave a fully
glycosylated cell surface-expressed PAR2 receptor, whereas being able efficiently to cleave synthetic peptides representing the nonglycosylated PAR2 N-terminal sequence or to cleave deglycosylated PAR2 expressed in cells (Compton et al., 2001).

Another important posttranslational modification that appears to regulate PAR function is palmitoylation of the receptor, as is reported for a number of other GPCRs (Qanbar and Bouvier, 2003). The palmitoylation of PAR1 at two C-terminal cysteine residues is important for the interaction of the activated receptor with the clathrin adaptor protein complex so as to affect receptor trafficking. That said, mutated PAR1 (CC/AA), lacking the cysteine palmitoylation sites, is nonetheless fully competent for signaling, as determined by an [3H]inositol phosphate accumulation assay (Canto and Trejo, 2013). By comparing the wild-type and palmitoylation-deficient CC/AA-mutated PAR1 constructs, it was determined that an enhanced constitutive internalization and lysosomal degradation of the receptor occurs in the absence of receptor palmitoylation. In the case of PAR2, however, the palmitoylation of the receptor occurs primarily at the C-terminal cysteine 361 and a palmitoylation-deficient C/A mutant receptor has greater cell-surface expression but a decreased ability to cause intracellular calcium signaling. However, the palmitoylation-deficient PAR2-expressing cells triggered greater and more prolonged ERK/MAP-Kinase phosphorylation compared with the wild-type receptor in a Goi-dependent manner (Botham et al., 2011). Given the ability of PARs to trigger biased signaling as described below, it will be of importance to assess the roles for posttranslational modifications in regulating PAR signaling when activated by different proteinases. For instance, it is not yet known if the receptor dynamics triggered by neutrophil or pathogen-derived proteinases that can stimulate biased signaling are the same as the regulation of PAR mobility and internalization triggered either by the canonical PAR activators, thrombin and trypsin, or by the tethered ligand-derived receptor-activating peptides.

E. Endogenous Proteolytic Regulators of Proteinase-Activated Receptor Function and Proteinase-Activated Receptor Signaling

The activation of PARs by enzymes in the tissue microenvironment can play key roles in inflammation and cancer (Fig. 1). These enzymes may also play a role in regulating the function of integrin and adhesion ADGR receptors (below). After the initial discovery of PAR1, PAR3, and PAR4 as thrombin-cleaved receptors and PAR2 as a trypsin-cleaved receptor, a key question was: what are the endogenous proteinases that regulate PAR activity in vivo? Understandably, thrombin can be seen as an important “physiological” blood stream-localized regulator for PARs 1 and 4; but what about the other coagulation proteinases? Furthermore, are there localized sites of synthesis of the coagulation proteinases, including thrombin, that release the enzymes only in the tissue microenvironment and not into the circulation? As opposed to thrombin, trypsin is thought of primarily as an intestinal digestive enzyme. Thus, which trypsin-like enzymes outside the intestinal lumen might be responsible for regulating PAR2? Clearly, the many diverse sites of expression of PARs in the body necessitate the existence of multiple endogenous proteinases to regulate PAR function in vivo. Thus, once the PARs were cloned and their mechanism of activation understood, studies were focused not only on the molecular pharmacology and pathophysiology of PAR signaling but also on identifying the enzymes that are responsible for PAR regulation in vivo in a tissue- and disease-specific context.

1. Coagulation Cascade Enzymes. After the discovery of PAR1 as a thrombin-activated receptor, additional enzymes in the coagulation cascade (Fig. 3, right) were evaluated for their ability to regulate the PARs. In particular, much evidence points to an important role for the factor VIIa/Xa complex as a regulator of PAR signaling. The activation of PAR2 by factor VIIa requires the presence of its cellular cofactor, tissue factor (Camerer et al., 2000). In addition to activating PAR2 directly, the tissue factor (TF)-VIIa complex converts the inactive zymogen factor X to the active factor Xa (Fig. 3), which also has the ability to activate PAR2 signaling (Camerer et al., 2000; Riewald and Ruf, 2001). It is unclear as to what role this response would play in platelet-regulated hemostasis, because PAR2 is not expressed on either human or rodent platelets. However, the activation of PAR2 by the TF-VIIa-Xa complex presumably has important roles in regulating vascular endothelial function and in tumor biology. PAR2 activation by the TF-VIIa-Xa complex drives breast cancer migration as assessed both in the Adr-MCF7 cell line (Jiang et al., 2004) as well as in the MDA-MB-231 and BT549 breast cancer cell lines (Morris et al., 2006).

PAR2 is expressed in both vascular endothelial and smooth muscle cells. Thus factor Xa-dependent activation of PAR2 can potentially trigger responses not only in intact vessels (Al-Ani et al., 1995; El-Daly et al., 2014) but also in coronary artery smooth muscle cells, aortic smooth muscle cells, adventitial fibroblasts, and aortic endothelial cells (McLean et al., 2001). Furthermore, although PAR2 has not been thought of as a target for thrombin activation, recent data show that at concentrations of thrombin that can be achieved in the setting of acute injury or tumor invasion (e.g., 10 to 25 U/ml), PAR2 can be activated directly both in cultured cells and in the endothelium of intact vessels to cause vasorelaxation (Mihara et al., 2016).

An additional regulatory mechanism for the Tissue Factor-VIIa-Xa complex activation of PARs involves the endothelial cell protein C receptor (EPCR). EPCR can
scaffold the interaction of factor X with TF-VIIa complexes (Disse et al., 2011) and also acts as a docking site for factor VIIa and activated protein C on endothelial cells (Ghosh et al., 2007; Sen et al., 2011). Since PAR2 and Tissue Factor-VIIa-Xa are co-localized in lipid rafts and caveolae, their proximity may act to segregate signaling spatially through this pathway (Awasthi et al., 2007). The endothelial protein C receptor (EPCR) is also reported to support endothelial PAR1 activation by factor Xa (Feistritzer et al., 2005; Schuepbach and Riewald, 2010). However, it is not clear whether this endothelium-localized activation of PAR1 by Factor Xa would complement the activation of PAR1 by thrombin which is also generated in step with Xa activation.

Once hemostasis has been restored, the fibrinolytic cascade is engaged to remove fibrin and disassemble the clot (Nesheim, 2003). In addition, upon binding thrombomodulin, thrombin causes the activation of protein C and the thrombin-activatable fibrinolysis inhibitor. This process leads to the generation of the anticoagulant, activated protein C (APC), and the inhibition of fibrin generation (Esmun, 2003, 2014; Ito and Maruyama, 2011). Activated protein-C is also an important regulator of PAR1 signaling but does not activate PAR2. The activation of PAR1 by the protein C pathway was first reported by Ruf and colleagues (Riewald et al., 2002) who found that APC activation of PAR1 can trigger protective anti-inflammatory, antiapoptotic signaling in endothelial cells (Riewald et al., 2003). Given that PAR1 signaling by thrombin itself in endothelial cells is reported to trigger proinflammatory responses that are the opposite of those stimulated by APC, Ruf and colleagues went on to explore these different PAR1 responses caused by activation of the same receptor by two different enzymes. Large-scale gene expression profiling studies showed a significant divergence in endothelial genes that are up- and downregulated by thrombin and APC (Riewald and Ruf, 2005). These studies showed that although thrombin generation is ongoing in the context of an injury, the concurrent activation of PARs by other coagulation cascade enzymes can signal in a different way via the same receptor (Mohan Rao et al., 2014; Mosnier et al., 2012; Schuepbach et al., 2012).

This kind of functional selectivity or biased signaling, well characterized for other G protein-coupled receptors (Kenakin and Miller, 2010; Kenakin, 2011, 2013; Kenakin and Christopoulos, 2013), is now known to apply to the activation of PARs by different proteinases (Hollenberg et al., 2014; Zhao et al., 2014a,b). Furthermore, as described above for the activation of PAR2 by the TT-Factor VIIa-Xa complex, membrane lipid raft microdomains are important orchestrators of the activated protein C/PAR1 signaling axis. Thus the special membrane locale where all components are brought together to enable APC-mediated activation of PAR1 can lead to site-selective biased signaling (Bae et al., 2007, 2008). In sum, several enzymes of the coagulation cascade including thrombin itself can activate PAR signaling. This activation can exhibit functional selectivity, depending on the proteinase and its microenvironment wherein PARs are cleaved.

2. Kallikrein-Related Peptidases and Proteinase-Activated Receptor Signaling. When PAR2 was first cloned, its expression was observed in unanticipated settings like the retina and prostate gland (Bohm et al., 1996b; Nystedt et al., 1994, 1995a). Because we were already aware of the expression of the prostate-specific antigen/KLK3 family of serine proteinases in the prostate, we suggested that the KLKs might take part in an autocrine signaling loop in this locale, where the localized production of active KLK proteinases might in turn signal to the coexpressed PARs. In support of this hypothesis, we were able to show that KLKs are indeed able to generate cell signaling by cleaving and activating the PARs (Oikonomopoulou et al., 2006). It was later shown that the KLKs exhibit variable pharmacological potencies for cleaving at the same tethered ligand site required for PAR activation and can trigger distinct G protein-coupled mechanisms depending on 1) which PAR member is the target (e.g., PAR1 versus PAR2 versus PAR4), 2) the localization of the KLKs or their upregulation in disease settings, and 3) their local concentration (Ramsay et al., 2008; Stefansson et al., 2008; Gratio et al., 2010; Oikonomopoulou et al., 2010a; Ramachandran et al., 2012a). For example, KLK7 (Stefansson et al., 2008) and KLK8 (Stefansson et al., 2008; Ramachandran et al., 2012a) are not able to cause PAR2 calcium signaling, whereas KLKs 5, 6, and 14 can induce prominent PAR2 signals. Furthermore, KLK8 and KLK14 are now known to disarm PAR1, shutting down its signaling potential by other proteinases (Oikonomopoulou et al., 2006; Ramachandran et al., 2012a). In contrast, KLK4 has been shown to induce signals via both PARs 1 and 2 (Ramsay et al., 2008). KLK-triggered PAR signaling has been found to play roles in many settings, including skin inflammation, as well as in neuronal pathologies and cancer (Oikonomopoulou et al., 2010b).

Specifically in inflammatory skin settings, a role has been established clinically for KLKs in the pathobiology of Netherton syndrome (Chavanas et al., 2000; Briot et al., 2009, 2010; Hovnanian, 2013). As reviewed elsewhere (de Veer et al., 2014), a signaling pathway has been discovered whereby KLK5 activates skin PAR2 to increase expression of proinflammatory cytokines and chemokines, such as tumor necrosis factor alpha and interleukin-8 in human keratinocytes. These data indicated that active KLK5 in the epidermis of Netherton syndrome patients triggers inflammation via PAR2, independently of the environmental stimuli and the adaptive immune system (Briot et al., 2009; Hovnanian, 2013; de Veer et al., 2014).
KLKs, and more specifically KLK14, were also shown to induce receptor inflammatory signals indirectly via the complement receptor for the anaphylatoxin C3a (known as C3AR) (Oikonomopoulou et al., 2013). This study pointed to a potential role for the KLK-C3AR receptor axis in subcutaneous inflammation using an in vivo model of paw inflammation. Interestingly, trypsin-responsive PAR2 signals in the same model are also known to trigger paw swelling, indicating a potential crosstalk of the two receptor systems in this model, namely PAR2 and the complement C3a receptor. The impact of this crosstalk in settings of pathology remains to be explored. Taken together, the four proteolytic cascades (coagulation, complement, kininogen, and KLK) can be seen to integrate signaling along with the PARs. Thus these proteolytic cascades can be considered as regulators of inflammation along with the PARs.

3. Neutrophil Proteinases as Regulators of Proteinase-Activated Receptor Function. In the setting of acute inflammation caused by traumatic or infectious events, the initial activation of the coagulation and complement cascades is followed rapidly by the influx of neutrophils. We have therefore been particularly interested in the potential roles of neutrophil proteinases in driving the acute inflammatory response. Locally secreted neutrophil proteinases such as neutrophil elastase, proteinase-3, and cathepsin-G can have a dramatic impact on tissue function in part by regulating PAR activity (Ramachandran et al., 2011; Mihara et al., 2013). As already mentioned, these enzymes can cause either unbiased or biased PAR signaling and can also disarm PARs by removing the N-terminal sequences that harbor the cryptic tethered ligand sequences. Although the neutrophil enzymes target PARs 1, 2, and 4, which are known to signal autonomously, they can also cleave the N-terminal domain of PAR3. However, the biologic impact of this cleavage of PAR3 is difficult to interpret.

One of the first studies that implicated neutrophil-derived enzymes as regulators of PARs hypothesized that in the setting of an acute lung inflammation, the large neutrophil influx and release of serine proteinases such as elastase and cathepsin-G might act on epithelial cell PAR2 (Dulon et al., 2003). Interestingly, in cultured A549 or 16HBE lung epithelial cells, elastase and cathepsin-G failed to trigger increases in calcium signaling but rather made the receptor refractory to subsequent activation by trypsin. Later studies also demonstrated that an elastolytic metalloproteinase from *Pseudomonas aeruginosa* similarly cleaved and disarmed PAR2, thereby preventing its subsequent activation by trypsin (Dulon et al., 2005). Based on molecular pharmacological studies with a PAR2 receptor that upon trypsin activation revealed mutated tethered ligand motifs (Al-Ani et al., 2004), we hypothesized that receptor cleavage, even in the absence of an intact canonical tethered ligand, might activate some signaling downstream of the receptor (Ramachandran et al., 2009). Indeed we found that the intact trypsin-revealed tethered ligand was necessary for coupling to the calcium signaling response but was not a requirement for triggering MAPK responses from PAR2 (Ramachandran et al., 2009). Extending this line of thought we investigated whether an enzyme such as neutrophil elastase, which cleaves downstream of the classic tethered ligand revealing site, might also trigger some but not all signaling downstream of PAR2. We were able to demonstrate that neutrophil elastase is a biased agonist for PAR2 and stimulates p42/44 MAPK activation while disarming the calcium signaling that would otherwise be caused by trypsin activation (Ramachandran et al., 2011). In subsequent work we showed that neutrophil elastase can also trigger comparable biased signaling responses via PAR1 (Mihara et al., 2013). The functional consequences of the neutrophil elastase-triggered biased signaling of PAR2 are still being worked out, but it can be pointed out that neutrophil elastase induces mucin production through PAR2 activation (Tull et al., 2012) and neutrophil-mediated activation of epithelial proteinase-activated receptors 1 and 2 regulates barrier function and trans-epithelial migration (Chin et al., 2008). More recently it was shown that PAR2 activation causes cross-sensitization of the transient receptor potential family channel TRPV4 to regulate a number of different cellular responses (Grant et al., 2007; Poole et al., 2013; Saieddine et al., 2015). Moreover, neutrophil elastase activation of PAR2 is able to support such crosstalk to affect cell function (Villegas-Mendez et al., 2007).

Although more is known about neutrophil elastase as a regulator of PARs, proteinase-3 can also cleave PAR1 (Tull et al., 2012; Mihara et al., 2013). In contrast to neutrophil elastase, which cleaves PAR1 downstream of the thrombin cleavage-activation site, proteinase-3 cleavage occurs upstream of the thrombin site and appears to trigger signaling that is distinct from the elastase-triggered response. As discussed above, EPCR acts as a cofactor to enable APC cleavage of PAR1. Proteinase-3 is shown to cleave EPCR, and this cleavage in principle can regulate APC signaling via PAR1, although this possibility has not yet been directly demonstrated (Villegas-Mendez et al., 2007).

In addition to the activation of PAR2 described above (Dulon et al., 2003), cathepsin-G can also activate PAR4 on platelets (Sambrano et al., 2000) and on colonic epithelial cells (Dabek et al., 2009). It is not yet known if the activation of PAR4 by cathepsin-G triggers signaling that is identical to activation by thrombin. However, in contrast with the activation of PAR4 on human platelets, activation of PAR4 by cathepsin-G on murine platelets does not support aggregation (Cumashi et al., 2001). Whether the observed disabling of murine platelet PAR3 by cathepsin-G contributes to this observed
difference is unclear. It can be noted that murine PAR3, which is disabled by cathepsin G, acts as a cofactor for PAR4 to facilitate PAR4 cleavage at low thrombin concentrations.

4. Matrix Metalloproteinases as Proteinase-Activated Receptor Regulators. In addition to neutrophil elastase, proteinase-3, and cathepsin-G, neutrophil-derived matrix metalloproteinases (MMPs) are also thought to play an important role in inflammation in locales ranging from tumor microenvironments to the central nervous system (Ardi et al., 2007; Deryugina et al., 2014; Han et al., 2014; Lee et al., 2014). MMPs 3, 8, and 9 have been singled out for attention in addition to MMP1, which can be released by platelets along with the other MMPs in an inflammatory situation. PARs have also been found to be targets of the MMPs. In particular, PAR1 activation by stromal MMP-1 promotes tumorigenesis in breast cancer (Boire et al., 2005) and promotes melanoma invasion and metastasis (Blackburn et al., 2009). In addition to acting directly on tumor cell PAR1, activation of human platelet PAR1 by MMP-1 also contributes to platelet activation and thrombogenesis (Trivedi et al., 2009). This MMP-1-stimulated PAR1 response can also be involved in the regulation of vascular integrity (Tressel et al., 2011). Thus the MMP-PAR1 signaling axis is of relevance to vascular regulation and angiogenesis in the setting of a tumor.

PAR1 activation by MMPs and thrombin is also implicated in stroke-induced neurotoxicity (Xue et al., 2006, 2009). Another MMP-PAR1 interaction has been found in cardiac myocytes and fibroblasts wherein beta-adrenergic stimulation causes an MMP13-mediated autocrine-paracrine activation of PAR1 (Jaffre et al., 2012). This cleavage unMASKs a novel tethered ligand sequence DPRS(42)|P(43)FLRN that differs from the one revealed by MMP1. MMP-1 specifically activates PAR1 by cleaving at LD(39)|P(40)RSFL rather than at the LDPR4(41)|S(42)FL thrombin cleavage site and does not cleave the other PARs. Of note, MMP1, along with MMP13, are biased agonists for PAR1, stimulating distinct downstream signaling in a way that differs from the activation of PAR1 by thrombin (Austin et al., 2013). Indeed, given the high expression of PAR1 in cardiac fibroblasts (Snead and Insel, 2012) and the potential impact of PAR regulation on cardiac pathophysiology (Fukunaga et al., 2006; Pawlinski et al., 2007; Sonin et al., 2013; Bode and Mackman, 2015), the MMPs along with the PARs can be thought of as joint therapeutic targets for PAR-associated cardiomyopathies. Thus, in an inflammatory situation, activation of PAR1 by both thrombin and MMPs can be complementary, stimulating PAR1 responses that are temporally and mechanistically distinct (Blackburn and Brinckerhoff, 2008). To date, the ability of the other neutrophil-derived MMPs that can play a part in the process of inflammation (MMPs 3, 8, and 9) to affect PAR signaling have not been explored directly, for example, in terms of their abilities to “disarm” PAR1 activation. In this inflammatory setting, the MMP inhibitors will also play a key role to regulate signaling. The upregulation of the TIMPSs would not only attenuate MMP-stimulated PAR signaling but would prevent remodeling of the extracellular matrix that can sequester cell-regulating polypeptides that in turn can act along with the PARs to stimulate fibrosis (Chambers and Scotton, 2012; Arpino et al., 2015). Thus both the MMPs and their TIMP inhibitors are of importance in regulating PAR signaling.

5. Cathepsins, Proteinase-Activated Receptor 2 Activation, and Inflammatory Pain. Added to the rapid influx of neutrophils at a site of inflammation, macrophages soon migrate into the area to augment the innate immune response and to contribute their secreted proteinases to the microenvironment. Inflammatory mediators can trigger the secretion of proteinases from macrophages (and microglial cells in the central nervous system), including the cysteine proteinase cathepsin-S, which has been detected in a number of inflammatory settings. Like other microenvironment proteinases that we have discussed so far, cathepsin-S can also signal via the processes outlined in Fig. 2, including mechanisms 4 (ion channel regulation), 5 (PAR activation), and 6 (release of a membrane-tethered agonist). In this regard, cathepsin-S has been documented to activate the epithelial sodium channel (ENaC) as discussed below (Haerteis et al., 2012a) and may well affect other ion channels. Further, cathepsin-S can release the membrane-tethered cytokine fractalkine from neurons, thereby contributing to pain sensitization (Clark and Malcangio, 2012). Of relevance to PAR2 signaling, it was recently found that, like neutrophil elastase, cathepsin-S can cleave and activate PAR2 at an N-terminal sequence site downstream from the canonical “R/S|SLIGLV—” trypsin activation site. Cathepsin-S cleaves PAR2 at E(56)|T(57) to reveal yet another novel noncanonical tethered ligand sequence, “TVFSVDEFSA—” (Zhao et al., 2014a,b). When unmasked by cathepsin-S, this newly discovered PAR2 tethered ligand stimulates signaling in a completely different way than that caused either by trypsin or by neutrophil elastase activation of PAR2. Thus, like the neutrophil elastase-revealed noncanonical tethered ligand, the one unmasked by cathepsin-S does not stimulate either calcium signaling or an interaction with beta-arrestin and does not cause receptor internalization. However, unlike the elastase-stimulated PAR2 response, cathepsin-S does not activate MAPKinase but rather stimulates adenyl cyclase and protein kinase A-dependent mechanisms to cause inflammation and hyperalgesia (Zhao et al., 2014a,b). This hyperalgesia results from a link between PAR2 activation and the enhancement of neuronal calcium flux through neuronal TRPV4 channels, as outlined above (Grant et al., 2007). The ability of this enzyme to regulate PAR1
signaling is very likely but has yet to be studied in any depth. Thus, overall, cathepsin-S can be seen to join a number of other microenvironment proteinases that can regulate inflammation and pain by the hormone-like mechanisms outlined in this chapter. What is evident from the sections above is that enzymes of the majority of the families outlined in Fig. 2 (metallo-, serine, threonine, and cysteine-proteinases), with very different mechanisms of catalysis and distinct target substrates, are all capable of triggering hormone-like signals. No doubt many other proteinases secreted in the course of an inflammatory response will have these kinds of actions.

F. Proteinase-Activated Receptor Regulation by Pathogen and Allergen-Derived Proteinases: Enzymes from Cockroaches, Molds, Dust Mites, and Trypanosomes

In addition to endogenously generated enzymes, we now know that pathogen-derived proteinases to which the lung, skin, and other tissues are exposed are also able to regulate PAR activity. The search for the antibody-generating allergens detected by sera from dust mite-allergic patients resulted in the identification of highly allergenic proteinases from the Dermatophagooides pteronyssinus (Der p) organism with tryptic, chymotryptic, and cysteine proteinase activities (Chua et al., 1988). A number of other diverse environmental allergens have since been found to contain proteolytic activity, including several species of molds (Shin et al., 2006), pollens (Gunawan et al., 2008), and the German cockroach (Wongtim et al., 1993). Recent work suggests that, similar to the house dust mite allergen, the proteinases in these many allergen sources may contribute to their allergenicity. Initially, the pathogenic properties of these enzymes were thought to be due to their ability to interact with IgE. No doubt, that interaction can explain some of the acute reaction of sensitized individuals to the allergens. However, we now believe that this pathogen-host interaction, which generates an inflammatory allergic response, is due in large part to the activation of proteinase-mediated signaling by one or more of the mechanisms outlined in Fig. 2. Work by us and by others has focused on identifying the serine proteinases present in such allergens and evaluating their ability to signal by cleaving and activating PARs (Adam et al., 2006; Arizmendi et al., 2011; Boitano et al., 2011). We suggest that this mechanism whereby pathogen-derived proteinases can enhance their inflammatory impact by the mechanisms shown in Fig. 2, including PAR regulation may be widespread. As an example, the sleeping sickness caused by the African trypanosome involves a PAR2-mediated mechanism, whereby the trypanosomal cysteine proteinase enables its passage into the central nervous system (Grab et al., 2009; Moxon et al., 2013). Thus pathogen-derived proteinases from organisms ranging from bacteria to insect allergens and tissue-invading parasites can use the mechanisms that trigger inflammatory signaling to their advantage. Therefore, for these pathologies, the proteinases themselves as well as their substrates like the PARs offer novel targets to consider for therapy.

IV. Adhesion G Protein-Coupled Receptors: A Proteinase-Activated Receptor-like Tethered Ligand Signaling Mechanism Driven by Receptors with Intrinsic Proteinase Activity

In Fig. 2, cell-matrix interactions, which regulate integrin “outside-in” and “inside-out” signaling, are singled out as one potential proteinase signaling target (mechanism 7). However, signaling via cell adhesion interactions is also now known to be regulated by a large superfamily of adhesion G protein-coupled receptors (aGPCRs) or ADGRs (recommended IUPHAR nomenclature (Hamann et al., 2015)), which are described in detail elsewhere (Yona et al., 2008; Arac et al., 2012b; Paavola and Hall, 2012; Langenhan et al., 2013; Liebscher et al., 2014a; Hamann et al., 2015). Surprisingly, ADGRs and the PARs share the tethered ligand paradigm of activation but via a very different ADGR autoproteolytic process. There is thus a common signaling theme between the PARs and the adhesion-activated receptors, namely, receptor cleavage that exposes a signal-generating tethered ligand. Furthermore, it is possible that in addition to the intrinsic proteolytic mechanism of ADGR activation, extracellular proteinases may also affect signaling by this receptor superfamily. Thus ADGRs like PARs may be targets for regulation by microenvironment proteinases.

The large family of ADGRs, including 33 human homologs, is the second largest family of GPCRs, related evolutionarily to the secretin family of GPCRs and expressed not only in all vertebrates but also in primitive animals and unicellular metazoans (Hamann et al., 2015). These receptors have been found to play key roles in diverse settings ranging from organ development to myelination and the innate inflammatory response, as detailed in the above-referenced review articles. The large extracellular domain that distinguishes these aGPCRs/ADGRs from other receptors comprises a unique heterodimeric structure generated by an autocatalytic cleavage that occurs during receptor biosynthesis and maturation. The surprising outcome of work on these intriguing receptors, for which few endogenous ligands have yet been identified, is that their mechanism of activation, as alluded to above, echoes the process used by the PARs, in terms of the unmasking of a tethered ligand (Fig. 7). The exposed tethered ligand of an adhesion-GPCR, termed the “Stachel” (German word for stinger) domain by Liebscher and colleagues, can drive receptor-G protein
coupling (Liebscher et al., 2014a,b; Hamann et al., 2015). Whether this mechanism applies to all of the ADGRs found in the human genome, apart from GPRs 126 (ADGRG6) and 133 (ADGRD1), for which the tethered ligand mechanism has been validated, remains to be determined.

The key to the activation of ADGRs lies in a common GPCR-Autoproteolysis-INducing domain (acronym: GAIN domain; Fig. 7), that triggers receptor cleavage during the process of endosomal maturation and transport to the cell surface (Arac et al., 2012a,b; Promel et al., 2013; Liebscher et al., 2014a; Hamann et al., 2015). Thus these receptors possess intrinsic proteolytic activity that is integral to their signaling mechanism. The GAIN domain is found in common with all of the human ADGRs and functions as an autoproteolytic fold including a short proteolysis site (termed the G protein proteolysis site, or GPS). The proteolytic cleavage mechanism at the GPS site echoes that of serine proteinases in which a basic histidine (or arginine in some ADGRs) participates with the hydroxyl of a serine or threonine (or a sulfhydryl of cysteine) to trigger a nucleophilic attack on a leucine or other amino acid carbonyl that results in peptide bond cleavage (Arac et al., 2012b). This cleavage leaves an N-terminal ADGR fragment tightly associated noncovalently with a C-terminal receptor domain as a heterodimer. Upon release of the N-terminal fragment from a docking site or upon binding its cognate ligand [e.g., binding of collagen III by GPR56/ADGRG1 (Luo et al., 2014)], the heterodimer is believed to dissociate, revealing the cell-attached receptor portion, representing the C-terminal receptor domain, as a heterodimer by microenvironment proteinases might be envisioned as a coordinated process. Proteolytic activation of ion channels by membrane-associated intracellular and secreted proteinases in the tissue microenvironment or by proteolytic cleavage downstream of the ADGR tethered ligand sequences could in principle either silence these receptors or, as for the PARs, reveal alternate tethered ligand sequences that might confer biased ADGR signaling. These topics merit attention in terms of our hypothesis that proteinases released into the microenvironment can in principle disrupt integrin or ADGR-matrix interactions and thereby regulate tissue signaling. This kind of process may be of particular importance in cancer tissues for the processes of oncogenesis and metastasis in which the ADGRs appear to play key roles. Clearly, the adhesion GPCR family represents a rapidly moving research target that merits close attention in the future. As for the PARs, the ADGR tethered ligand sequences and their receptor docking sites may offer clues for the development of therapeutic agents. However, as outlined above, unlike the ADGRs, the PARs depend on extracellular proteinases to unmask their tethered ligands and can therefore have distinct tethered ligands unmasked by different proteinases.

V. Proteolytic Regulation of Ion Channels

In addition to regulating PARs and the insulin receptor, proteolytic cleavage is also known to generate signals by cleaving ion channels. Proteinases are central modulators of ion channels in epithelial, endothelial, and neural tissues. Moreover, we now know that proteolytically mediated PAR activation can also regulate the function of members of the transient receptor potential vanilloid family members, TRPV1 and TRPV4. Thus proteolytic activation of PARs and ion channels by proteinases in the tissue microenvironment can be envisioned as a coordinated process. Proteolytic activation of ion channels is particularly important as an inflammatory sensory mechanism that constitutes an “early warning system” for the organism. This biologic process underlies the transduction of noxious and damaging signals during injury/trauma or infection and triggers protective cellular responses that initiate defense mechanisms to counterregulate inflammation in the tissue and promote resolution. In addition, the modulation of ion channels by proteinases was recently reported as a critically important signaling mechanism in the context of diseases such as cancer (Szabo and Bugge, 2011). We will not cover the indirect regulation of ion channels by intracellular proteinases activated in the course of GPCR signaling (for review, see Veldhuis et al., 2015), but will deal with the direct activation of these channels by membrane-associated intracellular and secreted proteinases.

A. Proteolytic Activation of L-type Calcium Channels

The voltage-gated L-type calcium channels control the influx of calcium in cardiac, vascular, and neuronal
cells. Blockers of these channels have been used for decades in the treatment of hypertension and cardiac arrhythmia (Hockerman et al., 1997). Importantly, these channels have been revealed to be crucial mediators of the fight or flight response, during which noradrenaline released from the sympathetic nervous system stimulates L-type calcium current through activation of $\beta$-adrenergic receptors, adenylyl cyclase, and phosphorylation by cyclic adenosine 3',5'-monophosphate-dependent protein kinase (PKA). This phosphorylation leads to an increase in cardiac and skeletal muscle contractility. The Cav1 pore-forming subunit that conducts the calcium current is a large protein (220–250 kDa) composed of four homologous domains. Both the amino- and carboxy-terminal domains of the channel face the intracellular milieu. Interestingly, the carboxy-terminal tail of the channel was reported to be proteolytically cleaved by the cysteine proteinase calpain (De Jongh et al., 1994) to form a fragment that remains covalently associated with the membrane-bound L-type channel (Gao et al., 2001), thereby negatively regulating its activity. This predicted proteolytic cleavage site is conserved in Cav1.1 in skeletal muscles (Hulme et al., 2005). Mechanistically, the current view suggests that the distal carboxyl terminal proteolytic fragment reduces L-type calcium current through an autoinhibitory mechanism caused by a direct association of the distal carboxyl tail with the Cav1 subunit (Gao et al., 2001; Hulme et al., 2006). Interestingly, Fuller et al. (2010) reported that Gs-coupled $\beta$-adrenergic receptor activation can lead to PKA-dependent phosphorylation of the Ser (1700) in the C terminus of Cav1.2 and trigger the dissociation of the inhibitory proteolytic fragment from the Cav1.2 subunit. This PKA-induced phosphorylation thus relieves channel autoinhibition. Furthermore, although several questions remain with regard to the precise underlying mechanism of this biologic process, in addition to the $\beta$-adrenergic receptor-driven phosphorylation event, the inhibitory action of the C-terminal proteolytic fragment also seems to be dynamically regulated by calcium influx through the L-type channel (Crump et al., 2013). This effect implies the contribution of a multitude of signaling molecules that converge toward the proteolytic regulation of the channel in a use-dependent manner. With regard to the identity of the proteinases involved in channel regulation, the calcium regulated neutral proteinases calpain I and II that have a catalytic mechanism in common with the cysteine-proteinase family, have been reported to play a pivotal role in the proteolytic processing of the carboxyl tail of the Cav1 channel (De Jongh et al., 1994). These enzymes, which can be regulated by increases in calcium influx, would thus contribute to PKA-dependent phosphorylation of the Ser1700 residue, which in turn disrupts the carboxyl terminus intramolecular interaction after proteolysis (Fuller et al., 2010).

**Fig. 7.** Signaling via adhesion G protein-coupled receptors (ADGRs). Autocatalytic processing of the ADGR at the GPCR proteolysis (GPS) site within the GPCR autoproteolysis-inducing (GAIN) domain results in a noncovalent complex of its N-terminal domain with a C-terminal receptor-associated GPS-domain sequence that, as for the PARs, can act as a tethered ligand (termed the Stachel or stinger domain by Liebscher and colleagues (Liebscher et al., 2014a;b; Hamann et al., 2015). Upon engagement of the N terminus of the ADGR with its adhesion target, the GPS domain is unmasked to act as a tethered ligand to activate cell signaling. It is possible that as for the PARs, ADGRs may also be regulated by microenvironment proteinases.
Of note, proteolytic cleavage of L-type channels has been found to differ with aging. Indeed, Michailidis et al. (2014) reported that age-dependent proteolytic processing of neuronal L-type channels by the calcium-dependent proteinase, calpain, can generate midchannels that remain functional at the plasma membrane but that exhibit biophysical properties differing from the full-length “uncleaved” channel. This difference in proteolytic processing by calpain may alter L-type channel-mediated neuronal functions such as synaptic plasticity and neurotransmission. Therefore, this process provides newly identified roles for proteolysis-dependent regulation of ion channels in physiologic states like aging.

B. Proteolytic Cleavage of the Amiloride-sensitive Epithelial Na⁺ Channel (ENaC/Degenerin Family): Epithelial Na⁺ Channel Activation by Proteolytic Enzymes

Among mechanisms that control sodium ion homeostasis, the absorption of sodium by epithelial tissue of the colon, the airways or the kidney must be finely regulated. The epithelial sodium channel (ENaC) is a critically important regulator of epithelial sodium transport. ENaC channels belong to the ENaC/degenerin family of non-voltage-gated ion channels that consists of ENaC, the neuronal mechanosensitive degenerins of nematodes (UNC, MEC, DEG, DEL), and the neuronal acid-sensitive ion channels (ASICs) (Gamper and Shapiro, 2007). Dysfunction and/or dysregulation of ENaC channels leads to numerous disorders such as Liddle syndrome, which is associated with excess reabsorption of Na⁺ and thus severe hypertension (Kellenberger and Schild, 2002, 2015). At the molecular level, ENaC is a heteromeric channel formed by assembly of structurally related α, β, and γ subunits (Canessa et al., 1994). Each subunit has two transmembrane domains (M1 and M2) and a pore region adjacent to the M2 domains of the three subunits (Fig. 8). Proteolytic cleavage of ENaC channels has been found to be the main mechanism of channel activation by increasing the open probability of the channel (Kleyman et al., 2009; Rossier and Stutts, 2009). Recent advances in the understanding of the mechanisms of ENaC channel activation have provided key information with regards to the site(s) of proteolytic cleavage within ENaC subunits and the identification of the proteinases that mediate channel activation (Rossier and Stutts, 2009).

Previous work using the trypsin inhibitor aprotinin suggested that the ENaC channel can be activated by serine proteinases (Chraibi et al., 1998; Vuagniaux et al., 2002). Biochemical studies have confirmed that the α and γ subunits of ENaC are processed by proteinases. Interestingly, proteolytic channel fragments can be detected in both cytosolic and plasma membrane fractions, indicating that proteolytic processing of the channel can result from intracellular proteinases, membrane-anchored proteinases, extracellular matrix-bound proteinases (metalloproteinases), and proteinases released into the extracellular milieu (Hughey et al., 2004a,b). A role for pathogen-derived inflammatory proteinases for ENaC regulation has yet to be evaluated in any depth. The first membrane-bound serine proteinase described to mediate ENaC channel activation was channel-activating proteinase-1, an ortholog of human prostasin. This proteinase belongs to the subgroup of S1 proteinases that are bound to the plasma membranes either by a C-terminal transmembrane domain (type I), an N-terminal proximal transmembrane domain (type II, or TTSP), or a glycosylphosphatidylinositol-anchored motif (Netzel-Arnett et al., 2003). In recent years, considerable advances have been made in understanding the molecular mechanisms of proteolytic activation of ENaC and in identifying the nature of the proteinases involved. The extracellular domains of the ENaC α and γ
subunits each contain two consensus recognition and cleavage sequences for furin and furin-like convertases. It has been found that proteolytic cleavage of these subunits occurs in intracellular compartments before channel anchoring at the plasma membrane. These findings suggested that proteolytic processing may be essential for maturation of the channel α and γ subunits en route to the cell surface (Hughley et al., 2004a). Therefore, the current view is that the unprocessed ENaC channel has a low open probability, thus triggering small sodium currents. This autoinhibitory mechanism is mediated by a disulfide-bonded loop in the α subunit. The serine proteinase furin cleaves the α subunit and thus removes autoinhibition during ENaC trafficking to the plasma membrane. This cleavage leads to an increase in channel open probability. Additionally, a second disulfide-bonded loop in the γ subunit can also be cleaved by furin to elicit partial activation of ENaC. Complete removal of the γ subunit autoinhibitory loop can be achieved by membrane-anchored prostasin at the cell surface, thus mediating full activation of the channel (Szabo and Bugge, 2011).

It is likely that other members of membrane-anchored and soluble serine proteinases present in the extracellular milieu may also activate furin-processed ENaC in a similar manner, because putative cleavage sites in the γ subunit of ENaC have been postulated for plasmin, elastase, tissue kallikrein (KLK1), and chymotrypsin (Adebamiro et al., 2007; Bruns et al., 2007; Passero et al., 2008; Haerteis et al., 2012b; Patel et al., 2012). Interestingly, proteinase-specific mechanisms of ENaC activation may provide differential degrees and modalities of channel activation in a tissue-dependent manner. For instance, Haerteis et al. (2014) identified two distinct cleavage sites for trypsin IV and trypsin I in γ ENaC. They showed that trypsin IV cleaves the γ subunit at Lys189, whereas trypsin I cleaves the channel at multiple sites. Given that trypsin IV is abundantly expressed in human epithelial cells of the kidney, the trypsin IV-mediated activation of ENaC may provide a unique mechanism of channel activation that could vary in pathophysiological states where trypsin IV expression/secretion is upregulated.

It should be noted that other groups of proteinases such as the ones that belong to the cysteine proteinase family have also been found to stimulate apical ENaC channels (Haerteis et al., 2012a). For instance, Haerteis and coworkers reported that purified cathepsin-S is able to cleave γ ENaC in a heterologous expression system. This work identified two valine residues (V182 and V193) that are essential for mediating cathepsin-S-dependent activation of the channel. Of importance, as already pointed out above, cathepsin-S can also cleave/activate PAR2 to cause biased signaling via adenylyl cyclase activation. Thus ENaC ion channel and PAR2 signaling can be coregulated by microenvironment proteinases like cathepsin-S. In summary, the ENaC channel can be taken to represent a “prototype” ion channel regulated in a complex way by both intracellular (e.g., furins) and extracellular proteinases (e.g., trypsin IV). Multiple extracellular enzymes appear to be able to regulate channel activity by cleaving the γ-subunit at distinct sites. This process may be of relevance not only for normal physiologic function but also for the effects of microenvironment proteinases produced by tumor cells and for the untoward impact of pathogen-derived proteinases.

C. Proteolytic Cleavage of the Acid-Sensing Ion Channels.

The acid-sensing ion channel-1 (ASIC1) is a hydrogen ion-gated channel that belongs to the ENaC/degenerin family. ASIC channels consists of a two transmembrane subunit that likely form trimer to generate a functional channel. ASIC1 is the first of four ASIC genes expressed in sensory neurons of the peripheral nervous system (Kellenberger and Schild, 2002, 2015). Regulation of ASIC1 channels by the serine proteinases trypsin, chymotrypsin, and proteinase K has been measured in heterologous expression systems where proteolytic processing inhibits acid-induced currents and lowers the pH of channel activation (Poiriot et al., 2004; Vukicevic et al., 2006). More recently, the epithelial transmembrane serine proteinase, matriptase, has also been implicated in modulating ASIC1 channel, specifically through three cleavage sites (Arg145, Lys185, and Lys384) located in the extracellular loop of the channel (Clark et al., 2010). In pathophysiological settings, ASIC channels are critically important for the response to ischemia and acidosis that induce endoplasmic reticulum stress and cell death. Along these lines, Su et al. (2011) documented a protective role for tissue kallikrein (very likely, KLK1) during ischemia/acidosis-induced injury. This protection may be mediated by the specific proteolytic regulation of the ASIC1a channel. Collectively, although serine proteinases modulate the ENaC/degenerin family, they exhibit opposite regulation of the different members of this family. Hence, it remains to be fully understood how proteolytic cleavage of ENaC subunits at some sites promotes an increase in channel open probability, leading to larger sodium current, whereas cleavage of other distinct sites inhibits the ASIC current.

D. Proteolytic Regulation of Voltage-Gated Ion Channels by β-Secretase and γ-Secretase

Voltage-gated sodium and potassium channels are critically important in cellular excitability by initiating and propagating action potentials along the axon of nerve cells. Early electrophysiological work studying voltage-clamped squid axons showed that pronase application removes the voltage-dependent sodium channel inactivation, probably through cleavage of the intracellular inactivation gate intrinsic to the channel
(Armstrong et al., 1973). Similar effects were found for the Shaker potassium channel. These studies provided the key basis to establish the “ball and chain” mechanism of mammalian Kv potassium channel inactivation (Hoshi et al., 1990). This mechanism involves the amino-terminal domain of the channel that forms the inactivation gate (ball) that is tethered to the channel pore upon depolarization, thus preventing the flow of potassium through the channel (Murrell-Lagnado and Aldrich, 1993). Proteolytic removal of the amino-terminal tail thus blocks Kv channel inactivation. More recently, voltage-dependent sodium channels have been shown to be proteolytically cleaved by the secretase family of proteinases, a multisubunit complex that cleaves transmembrane proteins. The α- and β-secretase (β-site of amyloid precursor protein-cleaving enzyme 1; BACE1) enzymes have been recognized as central proteinases implicated in the cleavage of amyloid precursor protein and the production of amyloid β (Aβ) peptides found in senile plaques in Alzheimer’s disease (Hardy and Selkoe, 2002). Interestingly, new evidence suggests that the aspartyl proteinases, β-secretase (BACE1) and γ-secretase can mediate proteolysis of voltage-gated potassium and sodium channels, respectively. The transmembrane topology of voltage-gated sodium channels is very similar to that of voltage-gated calcium channels (see above). Like most of the voltage-gated ion channels, their function and cellular trafficking is regulated by auxiliary subunits that associate directly with the main pore-forming α subunit (Savio-Galimberti et al., 2012; Catterall, 2014). Among these auxiliary subunits, the sodium channel-associated β2 subunit has been shown to be a substrate of BACE1, a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), and γ-secretase (Kim et al., 2005; Wong et al., 2005). Consequently, proteolytic modulation of β2 by BACE1 and γ-secretase activities promotes an increase in channel expression and thus neuronal membrane excitability (Kim et al., 2007; Hu et al., 2010; Huth et al., 2011). Other ion channels such as the cardiac hyperpolarization-activated cation channel are blocked by trypsin-induced proteolysis (Budde et al., 1994), and this inhibition may represent an important regulatory factor during heart failure (Chapman and Spinale, 2004). In fact, growing evidence has pointed to a role of matrix metalloproteinases (MMPs) as key players of left ventricle remodeling in patients with postmyocardial infarction (Sutton and Sharpe, 2000). Modulation of cardiac ion channels by MMPs in infarcted tissue may lead to functional cardiac remodeling and hence targeting MMP action on cardiac ion channels could represent an innovative and effective therapeutic approach.

In conclusion, the entire repertoire of ion channels modulated by proteolytic cleavage and importantly, the proteinases involved in this biologic process remains to be established. Attention is rarely given to the likelihood that the extracellular domains of many ion channels can be targeted by microenvironment proteinases in a way that may alter channel function. This area merits close attention in the future. Given the many roles that extracellular proteinases can play in cellular and tissue homeostasis, it will be crucial to understand how the proteolytic regulation and dysregulation of ion channels may participate in health and disease respectively. In addition to their regulation by endogenous proteinases, accumulating evidence indicates that ion channels may also be subject to regulation by pathogen-derived proteinases in the course of infectious processes. These mechanisms have begun to be described, for instance, in the context of enteritis or myocarditis after virus infections. The identification of the proteinases and ion channels substrates that participate in host-pathogen interactions may identify new targets for the development of clinically relevant therapeutic agents.

VI. Therapeutic Targeting of the Proteolytic Signaling System

Major roles for the proteinase-mediated signaling mechanisms outlined in Fig. 2 are to be found in the settings of acute and chronic inflammatory disorders, like tissue trauma, arthritis, colitis, asthma, cardiomyopathies, and neurodegenerative diseases as well as in cancer cell growth, invasion, and metastasis, as reviewed by us in more detail elsewhere (Ramachandran and Hollenberg, 2008; Ramachandran et al., 2012a). Thus the proteinases themselves along with their signal-generating substrates, including the substrate-triggered signal transduction pathways, can be considered as fruitful therapeutic targets. For instance, angiotensin converting enzyme (ACE) inhibitors and angiotensin AT1 receptor blockers have proved of enormous clinical benefit for the treatment of hypertension. This principle, targeting both proteolytic agonist production and the activated receptor, has also been used very successfully to regulate signaling stimulated by the coagulation pathway. For that purpose, coagulation inhibitors have been developed that either prevent the post-translational carboxylation-capacitation of prothrombin and factor VII (i.e., warfarin) or inhibit the catalytic activity of the enzymes that regulate signaling (e.g., dabigatran, a peptidomimetic thrombin inhibitor). Furthermore, inhibitors that block signaling by the PAR1 target of thrombin have also been developed (e.g., Vorapaxar). The PAR1 inhibitors have proved both of clinical utility and of value for assessing the role(s) of PAR1 in animal models of inflammatory disease. That said, the clinical use of Vorapaxar has been limited by its unexpected association with increased cerebral hemorrhage in a subset of treated patients (O’Donoghue et al., 2011; Wiviott et al., 2011; Goto and Tomita, 2014). Similarly, PAR2 antagonists...
that diminish inflammation in animal models are now available (Ramachandran et al., 2012b; Yau et al., 2013), but their clinical utility in humans has yet to be established. The challenge of considering the PARs or their activating proteinases for therapeutic purposes has been summarized in some depth elsewhere (Ramachandran, 2012; Ramachandran et al., 2012b).

The “take-home” messages from the literature dealing with proteinase signaling systems as therapeutic targets, as summarized in the above-quoted articles, are that 1) the proteinases clearly play key roles in a variety of inflammatory settings, but 2) targeting either the enzymes themselves or their signal-generating systems represents a substantial challenge. The therapeutic approach for proteinase-mediated signaling thus differs in complexity from the more straightforward development of conventional receptor antagonists. This challenge is due firstly to the common catalytic mechanisms shared between proteinases of a given class (e.g., serine proteinases with different substrate sequence targets) that render enzyme selectivity and potency difficult, and secondly, to the multiple proteinases of different classes that may cleave the same signal-generating substrate (but at slightly different sites) to yield a common inflammatory signal. Furthermore, even with very successful proteinase antagonists, like the angiotensin converting enzyme (ACE) inhibitors, which block the production of the hypertensive agonist (i.e., angiotensin II), there can be unanticipated side effects. Thus, with the ACE inhibitors, blocking angiotensin production comes hand-in-hand with the preservation of the activity of inflammatory peptides, i.e., the kinins.

This issue has surfaced with an appreciation of the angioedema encountered either idiosyncratically or in genetically susceptible individuals treated with ACE inhibitors (Roberts et al., 2012; Bas et al., 2015). The life-threatening angioedema is due to the lack of metabolism of plasma kallikrein-generated bradykinin, in keeping with the mechanism outlined in Fig. 5. Nonetheless, treatment of the hereditary form of this angioedema disorder has been developed from the use of both 1) a proteinase inhibitor that blocks the generation of kinins by plasma kallikrein (Kalbitor/Ecallantide, a Kunitz domain plasma kallikrein inhibitor) and 2) a GPCR inhibitor, Icatibant/Firazyr, that blocks the bradykinin B2 receptor (Bork, 2014). Thus the therapeutic strategy for ACE-induced angioedema deals on two levels with the mechanism illustrated in Fig. 5: 1) blocking the enzyme and 2) blocking the receptor. Of note, the successful enzyme inhibitor is not one that targets the enzyme catalytic site but rather an enzyme-selective Kunitz-domain inhibitor. This approach using endogenous enzyme-selective allosteric site inhibitors may prove to be more successful in the selective blocking of proteinase signaling than the design of catalytic site inhibitors. In sum, in keeping with mechanism 2 in Fig. 2, it has been possible to develop successful therapeutic agents for treating hypertension and hereditary angioedema. In a similar vein it has been possible to generate successful therapeutic agents for coagulation disorders by targeting both the enzyme (thrombin) and its target receptor (PAR1). For the complement system, recent work shows promise of developing effective C3a receptor antagonists that in principle can be coupled with inhibitors of the complement proteinase cascade. One can be optimistic that a comparable approach will succeed in dealing with other proteinase mediators of inflammation that may work by one or more of the mechanisms outlined in Fig. 2.

To place these successes in context, it is important to illustrate failures in targeting proteinases for therapeutic purposes. This issue is well illustrated by the search for potent matrix metalloproteinase inhibitors that can block MMP-induced cell migration and tissue invasion in the setting of cancer. Given the pathologic roles of MMPs in cancer and other diseases and the suitability of the enzymes as therapeutic targets, over 50 MMP inhibitors, were developed that blocked cancer progression in murine tumor models. However, although successful in the animal models of cancer, MMP inhibitors failed in clinical trials in humans. The failures were due to the as yet unexplained differences between humans and rodents in terms of the innate inflammatory responses that MMPs drive and because of insufficient knowledge about the distinct biologic roles played by the different MMPs. That said, there is a renewed understanding of MMP biology and there is indeed new hope that the suitably selective enzyme inhibitors may find a place in the therapeutic armamentarium (Vandenbroucke and Libert, 2014). For instance, it is now known that in addition to causing the degradation of extracellular matrix to enable cells to exit and metastasize from a tumor site, distinct MMPs can now signal to cells by activating PAR1 in quite different ways (Boire et al., 2005; Jaffre et al., 2012). Thus, effective therapy may depend on targeting both the enzyme and the effector signaling system that is activated enzymatically. One key will be to identify the model systems that best reflect the human pathology environment. In this regard, it is important to point out that across species, there is considerable variation in the abundances and types of proteinases in any given tissue. This point may be of importance for elements like mast cells, which can have a quite different complement of enzymes from one species to another. A further issue relates to the correlation (or not) of inflammatory response genes upregulated in rodents, compared with humans (Seok et al., 2013; Takao and Miyakawa, 2015). Thus animal models alone must be complemented by other approaches involving human-derived systems for predicting efficacy in human pathologies. This challenge is being met by the development of human tissue-derived “organoid” culture systems that retain some of the phenotypes of the parent intact organs.
VII. Future Perspectives

Given the complexities of signaling by proteinases outlined in Fig. 2 and as discussed in this review, it is important to ask: what does the future hold for targeting proteinases and their signal-generating substrates for therapeutic purposes? The answer in part is outlined in the previous section summarizing some of the successes and failures of the approach. That said, might there be some guiding principles based on the experience to date?

A. Targeting the Receptor

It can be suggested that, if the signaling process involves the generation of a peptide agonist that singles out a principle receptor as for the renin-angiotensin system, it makes good sense to target the relevant receptor. Thus, despite the unequivocal success of the angiotensin converting enzyme inhibitors for treating hypertension, the angiotensin AT1 receptor antagonists appear to be a pharmacologically more attractive and direct solution to the problem. Fortunately, unlike the production of prostaglandins that can cross over at multiple EP/FP/TP receptors, the proteolytically generated agonist peptides in general are more restricted in their receptor targets. Thus, if a single receptor system can be identified as responsible for the pathology, a receptor antagonist might be the best target. That avenue is one suggested choice for a therapeutic answer to the above question.

B. Targeting the Proteinase

In the complex setting of tissue inflammation or inflammation-related tumor growth and metastasis, multiple receptors triggered by proteolytic mechanisms are undoubtedly involved. As a well-known example of common agonists generating diverse signals, inflammation-generated prostaglandins can in principle be seen to act on multiple target receptors (e.g., EP/TP/FP). Thus, for proteinase-mediated signaling where multiple signaling targets can be affected, it may prove more efficient to target the ligand-generating enzyme rather than the ligands themselves or their receptors. With proteinases like those of the coagulation cascade, which are relatively substrate-selective, targeting the enzyme catalytic activity can clearly work, as evidenced by the therapeutic success of thrombin inhibitors and agents that block the posttranslational activation of the coagulation pathway enzymes. However, many other members of the serine proteinase and matrix metalloproteinase families have broad substrate specificities and have common catalytic sites that render substrate-selective enzyme inhibitors very challenging to develop. One fruitful alternative is to seek compounds that interact with unique allosteric enzyme sites that inhibit catalytic activity in an enzyme selective manner. However, as for the kallikrein-related peptidase family, these inflammation-involved proteinases can often work as “cascades” in keeping with the coagulation and complement cascades. Thus, inhibiting a single enzyme in an inflammatory setting might not prove therapeutically effective, because multiple enzymes in an inflammatory tissue microenvironment might provide a redundant/alternative pathway. All this is to say that dealing with a complex inflammatory setting may necessitate the inhibition of more than a single enzyme, for instance in the case of the multiple proteinases released by mast cells during an inflammatory response (Douaiher et al., 2014; Reber et al., 2014). Therefore, if mast cell stabilizers are not able to block the release of multiple proteinases into the microenvironment, a single proteinase inhibitor cannot possibly stop the signaling process. An approach to this situation is outlined in the following paragraph.

C. Potential Use of Broad-Spectrum Enzyme Inhibitors in a Restricted Setting over a Limited Time Frame: A Heretical Approach to Consider

Although an inflammatory setting may result in the activation of multiple proteinase families, the basic catalytic mechanisms of the inflammatory enzymes are relatively few in number (Fig. 2). Thus a majority of the proteinases secreted into the inflammatory microenvironment act via serine, cysteine, or metalloproteinase mechanisms. Therefore, a “heretical approach” might be to consider the localized and time frame-restricted administration of broad-spectrum inhibitors that target multiple classes and multiple enzymes of the same class. As an example, one can point to the limited, but clinically successful, use of an intranasal proteinase inhibitor for alleviating allergic rhinitis (Erin et al., 2006). Of note, the inhibitor, RWJ-58643, was able to target not only β-trypsin, but also trypsin and presumably many other trypsin family members released in the nasal cavity. This approach illustrates three important principles: first, the evaluation was done in humans and not other animal species, where different enzymes would very likely be active in the inflammatory setting; second, that multiple enzymes with a common catalytic mechanism can be blocked simultaneously and importantly; third, the inhibitors can be administered in a localized setting over a limited time frame. Clearly, the approach would not be appropriate for a systemic dosing of the inhibitors, which would certainly cause many “off-target” complications, and the approach takes advantage of the “fourth dimension” of therapeutics, namely the time factor. Thus, by limiting the time frame of enzyme inhibition to an acute exposure setting, it is possible to attenuate some of the rapid inflammatory signal pathways, leaving the signal pathways required for the resolution of inflammation at later time points unaffected. Thus the same signal stimuli that can be damaging early on may be required at a later stage for
the healing process. Thinking as a pharmacologist, one can use these principles for selected sites of drug delivery such as the skin, airway, gastrointestinal tract (oral cavity and intestinal lumen from esophagus to rectum), joint space, bladder, ear canal, and conjunctival sac, among others. Optimally, the agents used should be poorly absorbed, so as not to be systemically active, where we would cause untoward side effects. We suggest that by examining in detail the molecular pharmacology of the mechanisms of signal generation and by designing novel approaches to inhibit the enzymes either selectively or nonselectively as a family, it should be possible to design novel agents that can control the inflammatory process. To that end, it is hoped that the principles outlined in this review will lead to new therapeutic modalities to treat inflammatory disease in the future.

Acknowledgments

The authors are indebted to the editor and reviewers for their constructive comments that have led to a much improved quality of our text.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Ramachandran, Altier, Okonkomo, Hollenberg.

References


Proteinase-Mediated Signaling and Inflammation


Proteinase-Mediated Signaling and Inflammation

1139


Ramachandran et al. 2012


Proteinase-Mediated Signaling and Inflammation


