Protease-Activated Receptors: Contribution to Physiology and Disease

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healing, repair, and protection, whereas protease inhibitors and PAR antagonists can impede exacerbated inflammation and pain. Major future challenges will be to understand the role of proteases and PARs in physiological control mechanisms and human diseases and to develop selective agonists and antagonists that can be used to probe function and treat disease.

I. INTRODUCTION

Proteolytic enzymes are estimated to comprise 2% of the human genome (266). It is not surprising, therefore, that proteases participate in a diverse array of biological processes, ranging from degradation of dietary proteins in the lumen of the gastrointestinal tract to the control of the cell cycle. This review concerns the importance of proteolytic events at the cell surface in signal transduction, physiological control, and disease.

Proteases that are anchored to the plasma membrane or that are soluble in the extracellular fluid can cleave ligands or receptors at the surface of cells to either initiate or terminate signal transduction (Fig. 1). Thus cell-surface proteases can release or generate active ligands, or degrade and inactivate receptor agonists. For example, tumor necrosis factor (TNF)-α-converting enzyme or TACE cleaves the precursor of TNF-α at the plasma membrane, thereby releasing a soluble form of this proinflammatory cytokine. In a similar manner, angiotensin converting enzyme (ACE), which is also an integral membrane protein, converts angiotensin I to angiotensin II in the extracellular fluid to generate the principal active form of this hormone. In contrast, neutral endopeptidase degrades and inactivates the neuropeptide substance P (SP) in the vicinity of its receptors and thus terminates the biological effects of SP. Certain soluble and membrane-bound proteases cleave G protein-coupled receptors (GPCRs) at the cell surface to activate or inactivate receptors. For example, the coagulation factor thrombin cleaves protease-activated receptor 1 (PAR1) on platelets, which activates the receptor to induce platelet aggregation and hemostasis. Conversely, cathepsin G from neutrophils cleaves PAR1 at a different site from thrombin to

![FIG. 1. Mechanisms by which cell-surface proteolysis regulates signal transduction. Proteases can regulate signaling by cleaving ligands (A–C) or receptors (D and E). A: cell-surface proteases can induce shedding of membrane-bound signaling molecules; e.g., tumor necrosis factor-α (TNF-α)-converting enzyme or TACE, a member of the ADAM (a disintegrin and metalloproteinase) family of cell-surface proteases, liberates soluble TNF-α from the cell surface and thus releases a soluble cytokine. B: cell-surface peptidases can generate biologically active peptides; e.g., angiotensin converting enzyme or ACE converts the decapeptide angiotensin I (ANG I) to the octapeptide ANG II, the principal active form. C: cell-surface peptidases can degrade and inactivate neuropeptides; e.g., neutral endopeptidase (NEP) cleaves substance P (SP) at multiple sites to form inactive fragments. D: soluble proteases can cleave protease-activated receptors (PARs) to expose a tethered ligand that binds and activates the cleaved receptor; e.g., the coagulation factor thrombin cleaves PAR1 to activate the receptor. E: soluble proteases can cleave PARs to remove the tethered ligand, generating a disabled receptor; e.g., cathepsin G from neutrophils cleaves PAR1 to remove the tethered ligand and thereby prevent activation by thrombin.]

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generate a disabled receptor that cannot respond to thrombin, which could impede blood clotting. These proteolytic events are critically important for normal physiological control: the conversion of angiotensin I to angiotensin II is central to the reflex regulation of blood pressure and volume, and thrombin-induced aggregation of platelets is vital for normal hemostasis. However, these processes are also of great interest in understanding mechanisms of disease and for the development of effective therapies. Thus inhibitors of ACE are widely used to treat hypertension and congestive heart failure, and antagonists of PARs are being developed to treat thrombotic and inflammatory diseases.

The focus of this review is on the PAR family GPCRs. Four PARs have been identified by molecular cloning (Table 1). A wide range of proteases cleave and activate PARs, including proteases from the coagulation cascade, inflammatory cells, and the digestive tract. Receptor activation initiates an array of signaling events in many cell types with diverse consequences, ranging from hemostasis to pain transmission. We review the mechanisms by which cell-surface proteolysis activates PARs to initiate signal transduction, discuss the mechanisms that terminate signaling by PARs, review the importance of PARs in physiological control in major systems, and speculate on the contribution of PARs to disease. There are several comprehensive reviews of PARs (68, 108, 182, 217).

### II. PROTEASE-ACTIVATED RECEPTORS: MECHANISMS OF ACTIVATION BY CELL-SURFACE PROTEOLYSIS

#### A. Discovery and Structure

1. **PAR1**

PAR1, formerly known as the thrombin receptor, was cloned by two laboratories by the strategy of expressing RNA from thrombin-responsive cells of humans and hamsters in oocytes from *Xenopus* (233, 305). Clones were identified that encoded a protein of 425 residues with 7 hydrophobic domains of a typical GPCR. The deduced sequence of human PAR1 contained an amino-terminal signal sequence, and extracellular amino-terminal domain of 75 residues, and a potential cleavage site for thrombin within the-amino-tail (LDPR$^{41} S^{42}FLLRN$, where ↓ denotes cleavage) (Fig. 2).

2. **PAR2**

PAR2 was identified by screening a mouse genomic library using degenerate primers to the second and sixth transmembrane domains of the bovine neurokinin 2 receptor (214, 215). A clone was found that encoded a protein of 395 residues with 7 hydrophobic domains of a typical GPCR. The deduced sequence of human PAR2 contained an amino-terminal signal sequence, and extracellular amino-terminal domain of 75 residues, and a potential cleavage site for thrombin within the amino-tail (LDPR$^{41} S^{42}FLLRN$, where ↓ denotes cleavage) (Fig. 2).

### Table 1. A summary of PAR activating proteases, disabling proteases, activating peptides, localization, and phenotypes of PAR-deficient mice

<table>
<thead>
<tr>
<th>PAR1</th>
<th>PAR2</th>
<th>PAR3</th>
<th>PAR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activating proteases</td>
<td>Thrombin</td>
<td>Trypsin</td>
<td>Thrombin</td>
</tr>
<tr>
<td>FXa</td>
<td>Trypsin</td>
<td>Trypsin</td>
<td>Trypsin</td>
</tr>
<tr>
<td>APC</td>
<td>Tryptase</td>
<td>FVIIa</td>
<td>Cathepsin G</td>
</tr>
<tr>
<td>Granzyme A</td>
<td>FXa</td>
<td>MT-SP1</td>
<td>Gingipains-R</td>
</tr>
<tr>
<td>Gingipains-R</td>
<td>Proteinase-3</td>
<td>Proteinase-3</td>
<td>Proteinase-3</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Acrosin</td>
<td>Der P1 D9</td>
<td>Gingipains-R</td>
</tr>
<tr>
<td>Inactivating proteases</td>
<td>Cathepsin G</td>
<td>Plasmin</td>
<td>Cathepsin G</td>
</tr>
<tr>
<td>Elastase</td>
<td>Proteinase-3</td>
<td>Elastase</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleavage site AP</td>
<td>LDPR$^{41} S^{42}FLLRN$</td>
<td>SKGR$^{28} S^{29}LIGKV$</td>
<td>LPR$^{38} T^{39}FRGAP$</td>
</tr>
<tr>
<td>SPLLNR</td>
<td>SLIGKV</td>
<td>None</td>
<td>GYPGQV</td>
</tr>
<tr>
<td>TPLLNR</td>
<td></td>
<td></td>
<td>AYPGKF</td>
</tr>
<tr>
<td>Localization</td>
<td>Platelets (human), endothelium, epithelium, fibroblasts, myocytes, neurons, astrocytes</td>
<td>Epithelium, endothelium, fibroblasts, myocytes, neurons, astrocytes</td>
<td>Platelets (mouse), endothelium, myocytes, astrocytes</td>
</tr>
<tr>
<td>Phenotype of knockout</td>
<td>Partial embryonic lethality, vascular matrix deposition after injury</td>
<td>Impaired leukocyte migration; impaired allergic inflammation of airway, joints, kidney</td>
<td>Protection against thrombus formation/pulmonary embolism</td>
</tr>
</tbody>
</table>

References are provided in the text. PAR, protease-activated receptor; AP, activating peptide.
typical characteristics of a GPCR and with ~30% amino acid identity to human PAR1. The extracellular amino terminus of 46 residues contained a putative trypsin cleavage site SKGR\textsuperscript{34}S\textsubscript{35}LIGKV (Fig. 2).

3. PAR\textsubscript{3}

The discovery of PAR\textsubscript{2} provided the impetus for attempts to identify other receptors of this type. Indeed, the existence of additional receptors for thrombin was suggested by the observation that platelets derived from PAR\textsubscript{1}-deficient mice still responded to thrombin, whereas fibroblasts were unresponsive (64). PAR\textsubscript{3} was subsequently cloned using degenerate primers to conserved domains of PAR\textsubscript{1} and PAR\textsubscript{2} to screen RNA from rat platelets (135). The human and murine forms of PAR\textsubscript{3} were subsequently cloned, and human PAR\textsubscript{3} was found to share ~28% sequence homology to human PAR\textsubscript{1} and PAR\textsubscript{2}. Like PAR\textsubscript{1} and PAR\textsubscript{2}, PAR\textsubscript{3} is a typical GPCR with a thrombin cleavage site within the extracellular amino terminus at LPIK\textsubscript{38}T\textsubscript{39}FRGAP (Fig. 2).

4. PAR\textsubscript{4}

Two laboratories identified PAR-like sequences by searching expressed sequence tag (EST) libraries, and both groups subsequently used these sequences to clone a new GPCR, PAR\textsubscript{4} (143, 317). Human PAR\textsubscript{4} is a 385-amino acid protein, with a potential cleavage site for thrombin and trypsin in the extracellular amino-terminal domain: PAPR\textsubscript{47}G\textsubscript{48}YPGQV (Fig. 2). PAR\textsubscript{4} is ~33% homologous to the other human PARs, but with some distinct differences in the amino- and carboxy-terminal domains.

B. Proteases Cleave PARs to Expose a Tethered Ligand Domain

The general mechanism by which proteases cleave and activate PARs is the same: proteases cleave at specific sites within the extracellular amino terminus of the receptors; this cleavage exposes a new amino terminus that serves as a tethered ligand domain, which binds to conserved regions in the second extracellular loop of the cleaved receptor, resulting in the initiation of signal transduction (Fig. 2). There is no known function of the amino-terminal fragment of the receptor that is removed by proteolysis.

1. PAR\textsubscript{1}

The mechanism by which thrombin activates PAR\textsubscript{1} has been investigated in detail. Thrombin cleaves PAR\textsubscript{1} at
R_{41} \downarrow S^{42}FLLRN to expose the tethered ligand SFLLRN, which binds and activates the cleaved receptor, resulting in signal transduction. Several observations support this mechanism of activation. Mutation of the cleavage site prevents thrombin cleavage and signaling, indicating the importance of this site for activation of PAR_{1} (305). The general importance of proteolytic activation is indicated by the finding that replacement of thrombin site with an enterokinase site generates a receptor responsive to enterokinase (306). A synthetic peptide that mimics the tethered ligand domain (S^{42}FLLRNPNDKYEFP) directly activates intact PAR_{1}, without the requirement for hydrolysis by thrombin (305), and peptides as short as six residues (S^{42}FLLRN) are also fully active (253) (see sect. III). Such synthetic agonists, referred to as activating peptides (AP), are useful tools for investigating PAR functions. Direct physical proof that thrombin cleaves PAR_{1} is provided by the findings that exposure of platelets to thrombin reduces binding an antibody directed against the cleavage site of PAR_{1} but does not alter binding of an antibody directed to a domain distal to the cleavage site (212). Moreover, exposure to thrombin increases the electrophoretic mobility of epitope-tagged PAR_{1}, indicating a reduction of molecular weight by proteolytic cleavage (304).

2. PAR_{2}

Similar observations indicate that trypsin cleaves PAR_{2} at R_{34} \downarrow S^{35}LIGKV to reveal the amino-terminal tethered ligand SLIGKV in humans (213–215). Mutation of the trypsin site prevents trypsin cleavage and activation of PAR_{2}. Synthetic peptides corresponding to the tethered ligand domain (SLIGKV) activate PAR_{2} without the need for receptor cleavage. Exposure of cells to trypsin results in loss of immunoreactivity to an antibody against an amino-terminal epitope, which indicates that trypsin cleaves intact PAR_{2} at the cell surface (23).

3. PAR_{3}

Thrombin cleaves PAR_{3} at K_{38} \downarrow T^{39}FRGAP, and mutation of the cleavage site to one that would be resistant to thrombin prevents activation (135). Cleavage by thrombin exposes a new amino terminus (TFRGAP) that may interact with the receptor as a tethered ligand. However, in marked contrast to PAR_{1}, PAR_{2}, and PAR_{4}, synthetic peptides corresponding to this putative tethered ligand do not activate PAR_{3}. The reason for this discrepancy is unknown, although differences in affinity, steric hindrances, and the possibility that cleavage releases conformation of the receptor constrained by the uncleaved amino-terminal region could explain these unexpected results. Another unexpected and unexplained observation is that mouse PAR_{3} is unable to signal when expressed alone, without other PARs (143).

4. PAR_{4}

Thrombin and trypsin cleave PAR_{4} at R_{47} \downarrow G^{48}YPGQV, and peptides corresponding to the tethered ligand domain GYPGQV can directly activate PAR_{4} (143, 317). Mutation of the cleavage site prevents activation by thrombin and trypsin, but not by the synthetic peptide, which confirms the importance of proteolytic cleavage for receptor activation.

C. Protease Binding to PARs Facilitates Cleavage and Activation

Thrombin can activate PAR_{1} and PAR_{3} by a two-step mechanism: first the protease binds and then it cleaves the receptor (Fig. 3A). The process of binding and activation has been most thoroughly studied for thrombin and PAR_{1} (306). The extracellular amino terminus of human PAR_{1} contains a sequence of charged residues (1^{53}KYEPF^{56}) that is distal to the thrombin cleavage site. This charged domain binds to an anion binding site on thrombin, thereby temporarily concentrating the protease at the surface of the receptor. This negatively charged region of PAR_{1} resembles a domain of the leech anticoagulant hirudin, which inhibits thrombin by binding its anion site. The importance of the hirudin-like domain is emphasized by the finding that its deletion markedly diminishes the capacity of thrombin to activate PAR_{1}, whereas substitution of this region with the corresponding domain of hirudin allows a full recovery of activity. Moreover, γ-thrombin, which lacks an anion site, is 100-fold less potent than α-thrombin, which has the site, in cleaving PAR_{1} at the activation site (25). Furthermore, platelets respond to low concentrations of α-thrombin but not γ-thrombin, because the latter cannot bind to the PAR_{1} (260). Protease binding thereby increases the efficiency of activation, probably by concentrating the protease on the surface of the receptor or by altering the conformation of the receptor to facilitate cleavage. PAR_{3} also contains a hirudin-like site (FEEPF) that is distal to the thrombin cleavage site, which interacts with thrombin (135). Thus γ-thrombin is 100-fold less potent than α-thrombin for activating PAR_{3}, and alanine substitutions within the hirudin site of PAR_{3} attenuate activation of PAR_{3} by thrombin. PAR_{4}, in contrast to the other thrombin receptors, lacks a hirudin-like binding site for thrombin (143, 317). It is for this reason that both α-thrombin and γ-thrombin can activate PAR_{4} with similar potency (317). The lack of a thrombin binding site also accounts for the observation that α-thrombin activates PAR_{4} with a potency that is 50-fold less than for activation of PAR_{1}. Thus PAR_{4} is a low-affinity receptor for thrombin, whereas PAR_{3} and PAR_{4} are high-affinity thrombin receptors. Protease binding sites have not been identified for other PARs.
There Are Functional Interactions Between PARs

A common theme of signaling by GPCRs is that there are frequently several different receptors for a single ligand and often several ligands for one receptor. Thus thrombin can activate PAR1, PAR3, and PAR4, with different potencies, and trypsin, trypase, and certain coagulation factors can activate PAR2. This complexity becomes particularly interesting because PARs are frequently coexpressed, and interactions between receptors in the same cell can have important functional consequences.

1. Dual receptor systems

Human platelets express two thrombin receptors: PAR1 and PAR4. There are marked differences in the mechanisms of activation and inactivation of these receptors that have important consequences for thrombin signaling to platelets. PAR1, by virtue of a hirudin-like site, is able to bind thrombin and thus responds to low concentrations of enzyme. PAR4 lacks the hirudin site and can respond only to high thrombin concentrations (142). However, whereas PAR1 responses are rapidly shut-off, probably as a result of phosphorylation of residues in the carboxy terminus and uncoupling from G proteins (see sect. iv), PAR4 responses are sustained and thus desensitize slowly (263). The coexpression of two receptors with different potencies and kinetics of desensitization may have important functional consequences. Thus PAR1 mediates rapid and transient increases in intracellular Ca^{2+} concentration ([Ca^{2+}]_{i}) in human platelets to low concentrations of thrombin, whereas PAR4 mediates delayed and sustained increases in [Ca^{2+}]_{i} to higher thrombin concentrations (70). This prolonged signal is important for the late phases of platelet aggregation. Similar dual receptor systems probably exist on other cells with important functional consequences.

2. PAR3 as a cofactor for PAR4

In some instances, protease binding to one receptor can facilitate cleavage of another receptor that is expressed on the same cell. This appears to be the situation...
for PAR$_3$ and PAR$_4$, the thrombin receptors on murine platelets (mouse platelets lack PAR$_1$) (199) (Fig. 3B). Observations in mice deficient in PAR$_3$ indicate that PAR$_3$ is required for the response of platelets to low but not to high concentrations of thrombin, which is attributable to PAR$_4$. These findings could be explained by the fact that PAR$_3$ contains a thrombin binding site, and can thus respond to low concentrations of thrombin (135), whereas PAR$_4$ lacks a binding site and thus mediates responses to high thrombin concentrations (143, 317). However, the situation is complicated by the finding that murine PAR$_3$ expressed in COS cells does not signal, for reasons that are not fully understood. This finding suggests that, in murine platelets, PAR$_3$ somehow facilitates the activation of PAR$_4$ by low concentrations of thrombin. In support of this hypothesis, the potency of thrombin signaling to PAR$_4$ in COS cells is increased 6- to 15-fold by coexpression of PAR$_3$, whereas responses to PAR$_4$ AP are unaffected (199). Moreover, the expression of a construct of the amino terminus of PAR$_3$ (including the hirudin-like site) attached to the transmembrane domain of CD8 (to anchor the construct at the cell surface) with PAR$_4$ also facilitates thrombin signaling. Together, these findings suggest that PAR$_3$ is a cofactor for PAR$_4$ in murine platelets: the hirudin-like site of PAR$_3$ binds and concentrates thrombin at the cell surface, and thereby promotes thrombin cleavage of PAR$_4$. At high concentrations, thrombin can directly cleave and activate PAR$_4$, even though it lacks a thrombin binding domain. This novel mechanism of cooperative interaction between PAR$_3$ and PAR$_4$ adds to the complexity of interactions between GPCRs.

3. Intermolecular PAR signaling

GPCRs can form homodimers and heterodimers with important consequences for signal transduction. Although the principal mechanism of PAR activation is intramolecular (i.e., the unmasked tethered ligand binds to the cleaved receptor), there are several examples of intermolecular interactions between different PAR molecules. Intermolecular signaling, by which a cleaved receptor can activate an uncleaved receptor, was first demonstrated for PAR$_1$ (43). The approach was to express the amino terminus of PAR$_1$ (including the tethered ligand domain) attached to the transmembrane domain of CD8 (to anchor the fragment at the cell surface) with truncated PAR$_1$ lacking the amino terminus, either alone or together. When expressed alone, there was no response to thrombin. However, when coexpressed, cells were able to respond to thrombin, suggesting that thrombin cleaves the amino terminus of PAR$_1$ to reveal the tethered ligand domain, which then interacts with the truncated receptor, which transduces the signal. Thus at least in a reconstituted system, it is possible for the tethered ligand of cleaved PAR$_1$ to activate an uncleaved receptor. There is also evidence of intermolecular signaling between different PARs (Fig. 3C). Peptides corresponding to the tethered ligand of PAR$_1$ (SFLLRN) can also activate PAR2, but not vice versa (21). To examine intermolecular signaling, a signaling defective mutant of PAR$_1$ and wild-type PAR$_2$ were expressed alone or together (218). When expressed alone, neither receptor responded to thrombin, but when coexpressed, there was a robust thrombin response. Moreover, in endothelial cells that naturally express PAR$_1$ and PAR2, a PAR$_1$ antagonist blocked only 75% of the response to thrombin, whereas desensitization of PAR$_2$ blocked the remaining 25% of the response. These results suggest that the tethered ligand domain of PAR$_1$ can interact with uncleaved PAR$_2$ to transduce the signal. This novel form of intermolecular signaling between different PARs clearly requires the close association of receptors at the cell surface, which could be influenced by levels of expression or by anchoring proteins that may affect mobility of receptors in the membrane.

E. Protease Binding to Other Membrane Proteins

Can Facilitate PAR Cleavage and Activation

As discussed, thrombin binding to a PAR can facilitate cleavage and activation of that receptor or a different PAR. In addition, binding to nonreceptor proteins can concentrate proteases at the cell surface and thereby facilitate PAR activation. For example, glycoprotein I$_{b}$a$_{2}$ is a cell-surface platelet protein with a high-affinity binding site for $\alpha$-thrombin. Disruption of this interaction impedes the capacity of $\alpha$-thrombin to cleave PAR$_1$ and cause aggregation of platelets, suggesting that glycoprotein I$_{b}$a$_{2}$ is a cofactor for PAR$_1$ that promotes PAR$_1$-mediated platelet aggregation (84). Thrombin may also interact with other proteins on platelets (118).

This theme of anchoring proteins serving as cofactors for proteolytic activation of PARs is particularly important for signaling by coagulation factors (F), notably FVIIa and FXa that act upstream of thrombin (reviewed in Refs. 68, 240). Coagulation proceeds by two processes: an extrinsic and an intrinsic pathway. The extrinsic pathway is initiated by tissue damage and requires tissue factor (TF) or thromboplastin, an integral membrane protein that is normally expressed by extravascular cells (e.g., myocytes, keratinocytes), and which is expressed by endothelial cells and monocytes during inflammation. During coagulation, TF binds FVIIa, and the TF-FVIIa complex interacts with the zymogen FX to form active FXa. FXa then interacts with FVa, a cofactor for FXa-mediated conversion of prothrombin to thrombin. Thrombin plays a critical role in hemostasis by converting fibrinogen to fibrin and by cleaving PARs on platelets to induce aggregation. The intrinsic pathway is triggered by exposure of blood to collagen underlying damaged endothelium, and
begins with conversion of FXII to FXIIa, which is catalyzed by kallikrein and kininogen. This conversion then initiates a cascade of events involving activation of FXI, FX, and converging with the extrinsic pathway and the FXa-dependent conversion of prothrombin to thrombin.

In addition to zymogen cleavage, FVIIa and FXa can signal to PARs. FVIIa signals to cells when allosterically associated with TF for full activity, in part through PAR1 and PAR2 (34) (Fig. 4A). Thus, although FVIIa, even at high concentrations, does not signal to Xenopus oocytes expressing PAR1 or PAR2, FVIIa robustly signals to oocytes expressing TF together with PAR1 or PAR2. Similarly, soluble FXa weakly activates PAR1, PAR2, or PAR4, but when FXa is associated with a complex comprising TF-FVIIa-FXa, it potently activates PAR1 and PAR2 (34). Moreover, although the TF-FVIIa complex can activate PAR2, it does so with far lower efficiency than the TF-FVIIa-FXa complex, in which locally generated FXa induces signaling. Thus FVIIa can signal to fibroblasts expressing PAR2 and TF with a potency of ~4 nM, but this concentration exceeds the estimated plasma concentration of FVIIa (50 pM). However, the potency of FVIIa is reduced by almost three orders of magnitude (to 8 pM) in the presence of FX. These results suggest the local formation of FXa at the plasma membrane results in PAR2 activation (34). The receptor that mediates FXa signaling depends on the cell in question. Observations made using cells prepared from PAR-deficient animals indicate that FXa signaling to endothelial cells is mostly mediated by PAR2 but also by PAR1, whereas PAR1 solely mediates FXa signaling to fibroblasts (35). These mechanisms of signaling by FVIIa and FXa may be of particular importance during septic shock when upstream coagulation proteases make a substantial contribution to the inflammatory response and when the expression of TF is up-regulated on several cell types.

The role of anchoring proteins in PAR signaling has also been extended to the anticoagulant pathway (Fig. 4B). Low levels of thrombin are normally present in the circulation, and when vessels remain intact these low levels of thrombin contribute to the anticoagulant protein C (APC) pathway. The endothelial protein C receptor (EPCR) binds protein C (PC), and thrombin converts PC to activated PC (APC). When APC is released from the EPCR, it acts as a circu-
lating anticoagulant protein by degrading FVα and FVIIa. However, when retained at the cell surface, the EPCR-APC complex promotes activation of PAR1 (238). Thus APC can signal to fibroblasts only when they coexpress EPCR and PAR1, and APC signaling to endothelial cells is blocked by an active site blocked form of APC, which competes for EPCR, and by antibodies that block PAR1 cleavage. Gene expression analysis of endothelial cells indicates that this mechanism of APC-induced activation of PAR1 mediates in large part the anti-inflammatory effects of APC. Thus PAR1 mediates the effects of low concentrations of APC on gene expression in endothelial cells. In addition to its anticoagulant activity, APC is an anti-inflammatory agent that reduces organ damage in animal models (111) and reduces mortality of patients with sepsis (20). PAR1 can contribute to the anti-inflammatory actions of APC. Thus APC prevents hypoxic-induced apoptosis of brain endothelial cells through transcriptionally dependent inhibition of tumor suppressor gene p53, regulation of Bax/Bcl2 proteins, and reduction of caspase-3 signaling (46). Blockade of PAR1 with an antibody against the activation site prevents APC-mediated cytoprotection in cultured endothelial cells, indicating a crucial role for PAR1 in this protection. In a model of focal ischemic stroke in mice, APC markedly reduces brain infarction volumes. This effect is attenuated in EPCR-deficient mice, and the neuroprotective role of APC is abolished by PAR1 blocking antibodies. Thus EPCR and PAR1 play a major role in the protective effects of APC.

F. Multiple Proteases Activate PARs

Although many proteases have been found to cleave and activate PARs in cultured cells, the capacity of a protease to signal in intact tissues depends on many factors. First, proteases must be generated or released in sufficient concentrations to activate PARs. Although the catalytic properties of proteases would ensure that even very low concentrations could eventually cleave all receptors on the surface of the cell, it is the rate of hydrolysis of PARs that determines the magnitude of the resulting cellular signal (137). Second, efficient hydrolysis and activation of PARs may require the presence of accessory cofactors, for example, TF and EPCR in the case of FVIIa-FXa and APC, respectively (34, 46, 238, 239). Third, the capacity of a protease to signal will depend on the availability of protease inhibitors that serve to dampen the effects of many proteases in vivo. Thus, although trypsin is a very potently activated PAR2 in cultured cells, trypsin inhibitors are widely expressed and may well limit the capacity of trypsin to signal in tissues. With these caveats in mind, many proteases have been identified that are capable, at least theoretically, of activating PARs.

1. Coagulation factors

Serine proteases of the coagulation cascade are perhaps the best characterized activators of PARs (68, 108, 240). As discussed in section II, proteases that mediate coagulation and anticoagulation by cleavingzymogens or active enzymes themselves can also signal to several cell types by cleaving and activating PARs. Thus thrombin activates PAR1, PAR5, or PAR4 at the surface of platelets, resulting in aggregation, which contributes to hemostasis (68). The TF-FVIIa-FXa complex signals by cleaving PAR1 and PAR2 on a variety of cell types, including endothelial cells, which is of particular importance in inflammation (34, 239). Thrombin can also exert anti-inflammatory effects that depend on the local generation of APC and consequent activation of PAR1 on endothelial cells (46, 238).

2. Pancreatic and extrapancreatic trypsins

Trypsins potently activate PAR2 and PAR4. There are at least three distinct trypsin genes in humans: trypsin I, II, and mesotrypsin; trypsin IV is a splice variant of mesotrypsin. The potential of trypsins to signal to cells by cleaving PAR2 or PAR4 depends on the release of the zymogen trypsinogen, the presence of enteropeptidase, which activates trypsinogen, and the existence of the large array of endogenous trypsin inhibitors. During feeding, trypsinosens I and II are secreted from the pancreas into the lumen of the small intestine, where they are activated by enteropeptidase. In feeding rats, luminal trypsin attains concentrations (1 μM) that are more than capable of strongly activating PAR2 at the apical surface of enterocytes (EC50 ~5 nM) (167). Pancreatic trypsinosens are also prematurely activated in the inflamed pancreas where they are released into the interstitial fluid and vasculature and could activate PAR2 in pancreatic acini, duct cells, or nerves (205). However, trypsins are widely distributed enzymes that are expressed by many extrapancreatic cells, including endothelial cells (160), epithelial cells, the nervous system (168), and in tumors (165, 166). However, despite this widespread distribution, almost nothing is known about the control of secretion or activation of extrapancreatic trypsins or their potential functions as PAR activators. Trypsin II isolated from conditioned medium from colon cancer cell lines can cleave and activate PAR2; since these cells also express PAR2 it is theoretically possible that trypsin II could regulate cells in an autocrine manner (6, 94). A trypsin-like serine protease purified from rat brain, designated P22, degrades matrix and can signal to cells by activating PAR2 (252). P22 could be of particular interest in brain injury, where there appears to be enhanced secretion. Trypsin IV is also a potential PAR activator. Trypsinogen IV is invariably coexpressed in epithelial cell lines, endothelial cells, and human colonic mucosa with PAR2, and trypsin IV cleaves...
and activates PAR2 and PAR4 (N. W. Bunnett, unpublished observations). Of particular interest, trypsin IV is resistant to most proteinaceous trypsin inhibitors that effectively inhibit trypsins I and II (146). Together, these results raise the intriguing possibility that trypsin IV can signal for prolonged periods in tissues by autocrine or paracrine activation of PAR2 and PAR4.

3. Mast cell proteases

There has been considerable interest in mast cell tryptase as an activator of PAR2. Tryptase is the most abundant protease of human mast cells; it comprises up to 25% of the total cellular proteins and is expressed by almost all subsets of human mast cells (38). However, there are distinct interspecies differences in the expression of proteases in mast cells (reviewed in Ref. 190). Thus, whereas tryptase appears to be abundant in the cells of rats and mice. Many of the proinflammatory and mitogenic effects of tryptase are mimicked by PAR2 APs, suggesting that tryptase exerts its effects through this receptor. However, there is some unresolved controversy about the effectiveness of tryptase as a PAR2 activator. On one hand, human tryptase from a variety of sources (purified from human lung, skin, and mast cell lines) can cleave PAR2 to expose the tethered ligand domain and signals to transfected cells as well as many cell types that naturally express PAR2 at physiological levels (2, 18, 19, 66, 67, 193, 196, 235, 254, 256, 268, 270). These signals appear to be mediated by tryptase, for they are abolished by selective inhibitors, and are PAR2 dependent because they are absent from nontransfected cells and are diminished by selective downregulation of PAR2. The general consensus of these studies is that tryptase is a PAR2 activator but that it is considerably less potent than trypsin. However, most of the preparations used in these studies likely contain several forms of tryptase. Human mast cells express at least five distinct tryptase genes: α, βI, βII, βIII, and transmembrane tryptase, and splice variants also exist. Thus the molecular form of tryptase responsible for these effects is unknown. Observations using recombinant tryptase are less clear. Although recombinant α and βII tryptase have been reported to activate PAR2 in BaF3 cells (193), another report found no activity of βI tryptase as a PAR activator (130). The reason for this discrepancy is not known, but there are several possible explanations. One possibility is that a combination of tryptases in the purified preparations is responsible for PAR2 cleavage and activation. Another is that a posttranslational modification of PAR2 influences tryptase activation. PAR2 contains sites for N-linked glycosylation: Asn30, 6 residues proximal to the cleavage and activation site, and Asn222 in extracellular loop II. The potency with which tryptase (but not trypsin) activates PAR2 is dramatically increased by mutation Asn30 by enzymatic deglycosylation, or by expression of PAR2 in glycosylation-defective cells (62, 63). Thus glycosylation of the receptor at a site close to the activation site markedly impairs the capacity of tryptase to signal. Although the reason for this finding is not known, glycosylation could impede access of the amino terminus of PAR2 to the active site of tryptase. Tryptase is a 134-kDa tetrameric protease in the form of a flat ring that is composed of four monomers whose active sites face the center of the ring (228). One can speculate that a large, glycosylated structure may not be accessible to the active sites. It will be important to determine whether PAR2 is similarly glycosylated in tissues and to know if there are mechanisms that deglycosylate the receptor. However, can tryptase activate PAR2 in vivo? On balance, the evidence is in favor of an important role for tryptase in PAR2 activation under conditions of inflammation and mast cell activation when large amounts of tryptase are released close to PAR2 expressing cells. Thus injected tryptase has proinflammatory and hyperalgesic actions in conscious mice that are not observed in PAR2-deficient animals (40, 296). Because tryptase is a large and poorly diffusible protease, it is likely then that tryptase signals in a paracrine manner to cells that are in close proximity to mast cells, such as sensory nerves that express PAR2, which participate in inflammation and pain (267, 270, 296). Other proteases from mast cells have not been shown to activate PARs, although chymase can suppress thrombin signaling to keratinocytes, suggesting that it disables PAR1 (254).

4. Leukocyte proteases

Proteases from leukocytes are released at sites of inflammation and may serve as PAR activators under these conditions. Neutrophils store a variety of proteases (cathepsin G, elastase, proteinase 3) in azurophil granules, which may be released on activation. Cathepsin G is released from activated neutrophils and causes aggregation of platelets. This effect of cathepsin G may be mediated by PAR4 (249). Thus cathepsin G increases [Ca2+]i in PAR4-expressing fibroblasts, PAR4-expressing oocytes, and human platelets, and a PAR4-blocking antibody inhibits its activation of platelets by cathepsin G and prevents Ca2+ signaling in platelets induced by activation of neutrophils. Thus cathepsin G may activate platelets and other cells at sites of injury and inflammation by cleaving PAR4. However, some of the effects of cathepsin G are not PAR mediated, since cathepsin G also signals to cardiac myocytes in a PAR-independent manner (245). Proteinase 3 is present in neutrophil secretory granules and at the cell surface and is also expressed by other inflammatory cells. Proteinase 3 cleaves peptide fragments of PAR2 at the activation site, and proteinase 3-induced Ca2+ responses in oral epithelial cells are suppressed by desen-
sitation of PAR2, suggesting that proteinase 3 is a PAR2 activator (292).

5. Cell-surface proteases

Given that anchoring proteins serve as cofactors that facilitate the capacity of certain proteases to activate PARs, is it possible that proteases that are themselves integral membrane proteins can activate PARs? One such protease, membrane-type serine protease 1 (MT-SP1), has been identified. MT-SP1 is a type II integral membrane protein with an extracellular protease domain (276). Analysis of the substrate specificity of MT-SP1 suggests PAR2 as a potential substrate, and both MT-SP1 and PAR2 are coexpressed at the surface of certain cell types (e.g., PC-3 cells). Indeed, solubilized MT-SP1 signals to oocytes expressing PAR2, but not to cells expressing PAR1, PAR3, or PAR4. It remains to be determined if the membrane-bound MT-SP1 can activate PAR2 under more physiological circumstances.

6. Nonmammalian proteases

An intriguing observation is that a number of nonmammalian proteases from insects, fungi, and bacteria have been found to signal to mammalian cells by cleaving and activating PARs. The dust mites Dermatophagoides pteronyssinus and D. farinae produce a series of proteases (cysteine proteases, trypsins, chymotrypsins, and collagenases) that are allergens in the airway and can cleave fragments of PAR2 at the activation site, and desensitization experiments suggest that the effects of these proteases on airway epithelial cells are mediated in part by PAR2 (273). Bacterial proteases can also signal through PARs. *Porphyromonas gingivalis* is a major mediator of periodontitis in humans, and bacterial arginine-specific gingipains-R (RgpB and HRgpA) have been implicated in this disease. RgpB can activate PAR1 and PAR2-transfected cells and signals to an oral epithelial cell line to induce release of the powerful proinflammatory cytokine interleukin-6 (178, 179). RgpB and HRgpA can also signal to transfected cells expressing PAR1 and PAR3, and low concentrations of both proteases mobilize Ca$^{2+}$ in platelets and induce aggregation with a similar potency to thrombin (180). Cross-desensitization studies and use of antibodies that block PAR cleavage and activation suggest that the effects of gingipains-R on platelets are mediated by PAR1 and PAR4. These interesting results reveal a novel mechanism by which bacteria influence mammalian cells and could explain an emerging link between periodontitis and cardiovascular disorders. Certain fungi are also allergens in the airway, and proteases from extracts of several species affect cytokine production from airway epithelial cells (147). Some of these effects are reminiscent of the activation of PAR2, although the protease and receptor that are principally responsible remain to be determined.

III. PROTEASE-ACTIVATED RECEPTORS: TRANSDUCTION OF THE SIGNAL

A. Tethered Ligands Interact With Extracellular Domains to Initiate Signal Transduction

Analyses of mutant and chimeric receptors and of analogs of APs have allowed identification of the critical residues of the tethered ligand domains that interact with binding domains of the PARs and which are thus essential for signal transduction. The consensus of these experiments is that conserved regions of extracellular loop II and the amino terminus interact with the tethered ligands of PAR1 and PAR2.

Interactions between the tethered ligand and PAR1 have been investigated by analysis of chimeras of human and Xenopus PAR1 (105). The tethered ligands of human (SFLRNR) and Xenopus (TRFRFD) PAR1 are specific for their respective receptors and thus discriminate between the receptors. Replacement of the extracellular amino terminus and the second extracellular loop of the Xenopus receptor with the corresponding domains of human PAR1 confers the chimera with selectivity for peptides corresponding to the human tethered ligand. Moreover, substitution of only two residues of Xenopus PAR1 with corresponding residues of the human receptor (Phe$^{37}$ for Asn in the extracellular amino terminus and Glu$^{260}$ for Leu in the second extracellular loop) also confers selectivity for the human peptide. Thus these two residues in the extracellular amino terminus and extracellular loop 2 are critical for interaction with the tethered ligand domain. Further mutational analysis of human PAR1 and study of analogs of the PAR1 AP suggest that Glu$^{260}$ of extracellular loop 2 interacts with Arg$^{23}$ of the tethered ligand SFLRNR (201). Substitution of eight residues from the second extracellular loop of the Xenopus receptor to human PAR1 generates a receptor that is constitutively active in the absence of ligand, suggesting that alteration of the conformation of extracellular loop II is sufficient to transduce a signal across the plasma membrane (202).

Interactions between the tethered ligand of PAR2 and the cleaved receptor have been similarly examined by studying chimeras of PAR2 and PAR3 (175). These studies also reveal the importance of extracellular loop II for the activation of PAR2. The important role of residues in extracellular loop II of PAR2 for interaction with tethered ligand peptides has also been revealed by the study of mutant receptors and analogs of the PAR2 AP.
acidic region (PEE) that is just distal to a highly conserved domain (CHDVL) makes an important contribution to determining the selectivity of PAR2 agonists.

B. Structure-Activity Relations of Tethered Ligand Domains

The observation that synthetic peptides corresponding to the tethered ligand domain of PAR1 are agonists for the receptor without the need for proteolysis had three important consequences. First, it enabled the use of synthetic peptides as probes for PAR function, thereby avoiding the sole use of proteases, which have many biological effects not related to PARs. These APs are now widely used to investigate the physiological functions of PARs in vitro and in vivo. Second, it permitted convenient structure activity studies of the tethered ligand domain through functional analyses of synthetic peptides (187, 253). Such studies have provided important information about critical residues of the tethered ligand and facilitated the development of more selective agonists (123). Third, analogs of the APs have been used as templates for the development of antagonists of PAR1 (9). However, there are certain caveats to the use of APs as PAR agonists. Unfortunately, APs are weak agonists compared with proteases, often requiring concentrations in excess of 1 μM to activate most PARs. The low potency of APs is mostly attributed to the inefficient presentation of these soluble peptides to the binding domains of the receptor, compared with the tethered peptide. In addition, these peptides are readily inactivated by proteolysis. Because APs are used at such high concentrations, they may have nonspecific effects that are unrelated to PAR activation. The use of control peptides, such as inactive scrambled peptides or reversed sequences, is thus essential, although these too can have activity (296). In addition, APs are not always specific for a particular PAR; for example, the PAR1 AP SFLLRN also activates PAR2 (21). Finally, there are no effective APs for PAR3.

The characteristics of PAR APs and their utility as templates for design of antagonists have been recently reviewed in detail (182) and are only summarized here. The first described PAR1 AP was a 14-residue peptide (305), although it was soon realized that the hexapeptide SFLLRN was fully functional (253). Although SFLLRN is a PAR1 agonist, it also activates PAR2 (21). However, replacement of Ser1 with Thr1 (corresponding to the sequence in Xenopus PAR1) generates an agonist (TFLLRN) that is selective for PAR1 and that does not activate PAR2. Analysis of analogs of SFLLRN in which individual residues are substituted for Ala (alanine scanning), together with site-directed mutagenesis of the tethered ligand, indicates that critical residues within this domain include Phe2, Leu4, and Arg5. Substitution of other residues can be tolerated, depending on the type of substitution. For instance, changes in Ser1 maintain function provided the amino group is maintained, whereas removal of the amino group abolishes activity. Detailed structure-function analyses of analogs of the PAR1 AP, together with analyses by NMR and other techniques, have facilitated the design of penta- and tetrapeptides with enhanced potencies. For example, H-Ala-(pF)Phe-Arg-Cha-hArg-Tyr-NH2 induces platelet aggregation with an EC50 of 10 nM, and an iodinated form of this peptide can be used in binding assays (100). A peptidomimetic antagonist of PAR1 has been developed that is selective for PAR1 over the other PARs (9). Such antagonists have been successfully used in non-human primates to suppress thrombus formation and vascular occlusion (88) and could be of promise to treat human disease. Analyses of analogs of the PAR2 AP have similarly identified the residues that are essential for biological activity. In general, the rat/mouse peptide SLIGKV is slightly more potent than the human agonist SLIGRL (21). Analysis by alanine scanning indicates that Leu2 and Arg5 are essential for activity. Replacing Ser1 or Arg5 with Ala also reduces activity, whereas substitution of Gly4 or Leu6 has only a slight effect on PAR2 activation. The PAR4 AP GYPGKF is neither as efficacious as thrombin in activating PAR4 nor as potent as APs for PAR1 or PAR2 in activating the corresponding receptors, and is thus of limited use for probing receptor function. However, the analog AYPGKF is ~10-fold more potent than GYPGKF and is as efficacious as thrombin in activating PAR4 (98). AYPGKF is also relatively specific for PAR4. This specificity depends on Tyr2, since replacement with Phe generates an agonist of PAR1 and PAR4.

C. PARs Activate Multiple Signaling Cascades

In common with most GPCRs, PARs couple to multiple signaling pathways and can thereby regulate many cellular functions. The mechanisms of PAR1 and PAR2 signaling have been extensively investigated and reviewed (89, 182).

1. Concentration-response relationships

The catalytic nature of PAR activation means that even a very low concentration of an enzyme would eventually cleave every receptor molecule at the cell surface. How then do proteases generate graded responses in cells? For neurotransmitter receptors, graded concentrations of agonists induce graded responses through differing degrees of receptor occupancy. For PARs, graded responses depend on the rate of receptor cleavage (137). Thus thrombin induces cumulative phosphoinositide hydrolysis that correlates with cumulative receptor cleavage, suggesting that each cleavage event generates a “quantum” of phosphatidylinositol signal. This signaling is
rapidly terminated despite the continued presence of an exposed tethered ligand, by mechanisms of desensitization that are discussed in section IV B. Thus cells sense the rate of receptor cleavage, which is related to the concentration of protease. In other words, high concentrations of thrombin rapidly cleave many PAR1 molecules, permitting the accumulation of inositol 1,4,5-trisphosphate (InsP$_3$) and signal transduction. Proteases that inefficiently cleave PARs could not rapidly generate a sufficient quantity of second messenger for signal transduction to occur.

2. PAR$_1$ signaling

Signal transduction commences with coupling of PARs to heterotrimeric G proteins at the plasma membrane. Considerable progress has been made in identifying the subtypes of G proteins that interact with PAR1, and the role of these subunits in signaling and physiological regulation has been studied in knockout mice (reviewed in Ref. 219). PAR1 interacts with several $\alpha$-subunits, in particular $G_{q11}\alpha$, $G_{12/13}\alpha$ and $G_{i}\alpha$, which accounts for the pleotropic action of its ligands (Fig. 5). A major pathway of coupling is through $G_{i}\alpha$ (131). $G_{i}\alpha$ plays an important role in coupling since thrombin signaling in fibroblasts and platelets that express PAR$_1$ is attenuated by the microinjection of $G_{i}\alpha$ antibodies (13, 16). Moreover, platelets derived from $G_{i}\alpha$-deficient mice exhibit markedly diminished thrombin-induced aggregation and degranulation (222). These mice have increased bleeding times, probably due to impaired thrombin signaling to platelets. $G_{q11}\alpha$ activates phospholipase C-β$_1$, leading to generation of InsP$_3$, which mobilizes intracellular Ca$^{2+}$, and diacylglycerol (DAG), which activates protein kinase C (PKC). Thus thrombin-stimulated InsP$_3$ generation and Ca$^{2+}$ mobilization are blunted in mutant fibroblasts that express low levels of phospholipase C-β$_1$ but normal levels of the other phospholipases (99). Thrombin-induced activation of phospholipase A$_2$ and phospholipase D is diminished in this cell line, suggesting that phospholipase C-β$_1$ activates PKC-α, which may be required for phospholipase A$_2$ and phospholipase D activation. Together, Ca$^{2+}$ and PKC activate numerous pathways, including Ca$^{2+}$-regulated protein kinases and mitogen-activated protein (MAP) kinases.

PAR$_1$ also couples to $G_{12}\alpha$ and $G_{13}\alpha$ in platelets and

**FIG. 5.** Summary of PAR$_1$ signal transduction. PAR$_1$ couples to $G_{i}\alpha$, $G_{12/13}\alpha$, and $G_{q11}\alpha$. $G_{i}\alpha$ inhibits adenylyl cyclase (AC) to reduce cAMP. $G_{12/13}\alpha$ couples to guanine nucleotide exchange factors (GEF), resulting in activation of Rho, Rho-kinase (ROK), and serum response elements (SRE). $G_{q11}\alpha$ activates phospholipase C-β (PLCβ) to generate inositol trisphosphate, which mobilizes Ca$^{2+}$, and diacylglycerol (DAG), which activates protein kinase C (PKC). PAR$_1$ can activate the mitogen-activated protein kinase cascade by transactivation of the EGF receptor, through activation of PKC, phosphatidylinositol 3-kinase (PI3K), Pyk2, and other mechanisms. Gβγ subunits couple PAR$_1$ to other pathways, such as activation of G protein receptors (GRKs), potassium channels (K), and nonreceptor tyrosine kinases (TK). [Modified from Coughlin (68).]
astrocytoma cells (10, 220). Thus thrombin stimulates incorporation of a GTP analog into G_{12\alpha} and G_{13\alpha} in platelets, and thrombin-stimulated DNA synthesis is blocked by microinjection of antibodies to G_{12\alpha} in astrocytoma cells. G_{12\alpha} participates in cytoskeletal remodeling by thrombin. Upon stimulation with thrombin, platelets change from a discoid to a spheroid shape and extrude pseudopodia, which is believed to be a prerequisite for full activation. Although platelets from G_{9\alpha}-deficient animals show impaired thrombin-induced aggregation and degranulation (222), they undergo normal shape changes in response to thrombin (163). The effects of thrombin on the shape of these platelets is mediated by G_{12/13\alpha} (163). G_{12/13\alpha} interacts with Rho guanine-nucleotide exchange factors (GEFs), permitting Rho-mediated control of cell shape and migration. For example, in platelets G_{12/13\alpha} mediate activation of Rho-kinase and myosin light-chain kinase, which participate in thrombin-induced shape changes. Fibroblasts from G_{13\alpha}-deficient mice show diminished migratory responses to thrombin, and these animals also exhibit impaired ability of endothelial cells to develop into an organized vascular system, resulting in intrauterine death (221). In human umbilical vein endothelial cells, PAR1 activators induce stress fiber formation, accumulation of cortical actin, and cell rounding (303). Inhibition of Rho partially attenuates thrombin-induced cell rounding, whereas dominant-negative Rac blocks the response to thrombin. Thus Rho is involved in the maintenance of endothelial barrier function and Rac participates in cytoskeletal remodeling by thrombin.

PAR1 also couples to G_{i\alpha} proteins, which inhibit adenylyl cyclase and suppress formation of cAMP. Activation of PAR1 in fibroblasts inhibits cAMP generation in a pertussis toxin-sensitive fashion, suggesting involvement of a G_{i}-like protein (132). Expression of a mutant of G_{i,2} in CHO cells suppresses thrombin-stimulated arachidonic acid release, suggesting that G_{i,2} couples PAR1 to cytoplasmic phospholipase A_{2} (315).

G_{\beta\gamma} subunits of heterotrimeric G proteins couple PAR1 to may other pathways, notably activation of phosphatidylinositol (PI) 3-kinase. PI 3-kinase thus links PAR1 to changes in cytoskeletal structure, cell motility, survival, and mitogenesis. For instance, in astrocytes, the effects of PAR1 agonists on activation of extracellular signal response kinases (ERKs) 1/2 and proliferation are strongly inhibited by wortmannin, which blocks PI 3-kinase (310).

There has been considerable interest in understanding the mechanisms by which PAR1 couples to the MAP kinase cascades, given the important mitogenic role of thrombin. Several MAP kinase “signaling modules” have been characterized in mammalian cells (reviewed in Ref. 314) with a common organization: a MAP kinase kinase (MAPKK), which in turn phosphorylates MAP kinase (MAPK), which in turn phosphorylates MAP kinase (MAPKKK), which in turn regulates multiple substrates in the cytoplasm and nucleus. The MAPK ERK1/2 module plays a critical role in cell proliferation and differentiation. Most information about the regulation of this module derives from study of tyrosine kinase receptors, such as the epidermal growth factor (EGF) receptor. EGF binding to its receptor results in receptor dimerization and autophosphorylation. The phosphotyrosine on the intracellular domain of the receptor binds through an SH2 domain to the adaptor protein Shc, which recruits the Grb2-SOS complex to exchange GDP for GTP on p21ras. This initiates a cascade of phosphorylation events: p21ras phosphorylates the serine-threonine Raf-1 kinase, a MAP kinase kinase kinase, which in turn phosphorylates MEK1/2 (MAP kinase kinase), which in turn phosphorylates ERK1/2 (MAP kinase). There are several mechanisms by which PAR1 can couple to this pathway. In astrocytes, PAR1 activation of ERK1/2 and proliferation depend on a pertussis toxin-sensitive pathway mediated by G_{\beta\gamma}, PI 3-kinase, and G_{12/13}, and a pertussis toxin-insensitive pathway involving PKC and rac (310). Another mechanism may involve transactivation of the EGF receptor. Indeed, activation of PAR1 induces phosphorylation of the EGF receptor in enterocytes, and PAR1-induced Cl^- secretion is suppressed by inhibition of EGF receptor kinase (30).

Possible mechanisms of transactivation include activation of the Ca^{2+}-dependent kinase Pyk-2, and PAR1 agonists activate Pyk-2 in endothelial cells (161). Additionally, some GPCRs activate matrix metalloproteases, which induce shedding of EGF receptor ligands from the cell surface (231). PAR1 can also activate the MAP kinase p38 module in fibroblasts by a mechanism involving EGF receptor transactivation by Src family kinases (246).

The observation that mitogenic effects of PAR1 activators require tyrosine kinase activity, yet PAR1 does not possess intrinsic tyrosine kinase activity, led to the investigation of the role of the janus family of tyrosine kinases (JAKs) in PAR1 signaling and mitogenesis. JAKs tyrosine phosphorylate STAT proteins (signal transducers and activators of transcription), which dimerize and translocate to the nucleus to direct gene transcription. In vascular smooth muscle cells, thrombin activates JAK2, resulting in nuclear translocation of the transcription factors STAT2 and STAT3 (183). Inhibition of JAK2 suppresses thrombin-induced ERK2 activity and proliferation, suggesting that JAK2 is upstream of the Ras/Raf/MEK/ERK pathway.

3. PAR2 signaling

Less is known about the signaling mechanisms of PAR2 than for PAR1. Activators of PAR2 stimulate generation of InsP_{3} and mobilization of Ca^{2+} in PAR2-transfected cell lines, enterocytes, keratinocytes, myocytes,
neurons, astrocytes and tumor cells (24, 66, 67, 167, 213, 214, 251, 290). Therefore, it is likely that PAR2 couples to $G_{q,\alpha}$. Indeed, PAR2 signaling is unaffected by pertussis toxin in PAR2-transfected cells and in enterocytes, suggesting that PAR2 does not signal through $G_i$ proteins (86). In enterocytes and transfected epithelial cells, activation of PAR2 also stimulates arachidonic acid release and rapid generation of prostaglandins $E_2$ and $F_{1\alpha}$, suggesting activation of phospholipase $A_2$ and cyclooxygenase-1 (167). PAR2 activators also strongly activate MAP kinases ERK1/2 and weakly stimulate the MAP kinase p38, although c-jun amino-terminal kinase is not activated (15, 86, 319).

Recent observations indicate that $\beta$-arrestins play a major role in PAR$_2$-induced activation of ERKs. $\beta$-Arrestins are cytosolic proteins that interact with activated GPCRs at the plasma membrane and in the cytosol (see sect. iv). Activation of PAR$_2$ stimulates the assembly of a MAP kinase signaling module, a large-molecular-weight complex containing PAR2, $\beta$-arrestins, raf-1, and activated ERK1/2, which forms at the plasma membrane or in early endosomes (86) (Fig. 6C). The module retains activated ERK1/2 in the cytosol where they cannot induce proliferation. However, in cells expressing a mutant PAR2, which is unable to interact with $\beta$-arrestins, the module is unable to form. In these cells PAR2 agonists activate ERK1/2 by

![Diagram of desensitization, downregulation, and trafficking of PARs](http://physrev.physiology.org/)
alternate pathways that allow nuclear translocation and stimulation of proliferation. Therefore, β-arrestins are molecular scaffolds that promote the formation of an ERK1/2 module at the plasma membrane or in endosomes. The scaffold retains activated ERKs in these locations, where they may regulate cytosolic targets. PAR2 agonists also activate the NFκB pathway in keratinocytes and myocytes, emphasizing the importance of PAR2 in inflammation (28, 145).

IV. PROTEASE-ACTIVATED RECEPTORS: TERMINATION OF THE SIGNAL

A. Cell-Surface Proteolysis Can Disable PARs

Proteases that remove or destroy the tethered ligand or cleave the binding domain in extracellular loop II would generate receptors that are unresponsive to activating proteases. Many proteases can disable PARs in this manner (Table 1). However, the same criteria that should be applied to consideration of the physiological relevance of activating proteases (see sect. aF) must also be applied to any potential inactivating enzymes. Disabling proteases may serve to dampen signaling by activating proteases and could thereby be an additional mechanism for terminating protease signaling. A portion of proteolytically activated PAR1 can recycle to the cell surface in some cell types, although it is usually targeted for degradation (129). Proteolytically activated and recycled PAR1 can continue to signal at the cell surface (283). In this case, proteases that cleave or remove the exposed tethered ligand would arrest signaling.

Proteases from inflammatory cells, including neutrophils and mast cells, can cleave and disable PARs. Neutrophil cathepsin G, elastase, and proteinase 3 cleave PAR1 to remove the activation site and thereby abolish thrombin signaling (197, 236). PAR1 AP still signals to cells exposed to these proteases, suggesting that the binding domain is preserved. Chymase, an abundant mast cell protease, also renders keratinocytes unresponsive to thrombin, suggesting that it too inactivates PAR1 (254). Elastase and cathepsin G cleave PAR2 in transfected cells and airway epithelial cell lines, removing amino-terminal epitopes and thereby generating receptors that are unresponsive to trypsin but normally responsive to PAR2 AP (95). However, elastase and cathepsin G can signal to fibroblasts to induce release of interleukin-8 and monocyte chemoattractant protein 1, possibly by activation of PAR2 (291). Cathepsin G and elastase also abolish signaling by thrombin to PAR3-transfected cells, and thus disable PAR3 (74). Interestingly, this process of inactivation occurs without the loss of binding of monoclonal antibodies that recognize sites flanking the tethered ligand domain, suggesting inactivation does not involve removal of the tethered ligand. The mechanism of this inactivation is unknown but could involve cleavage of tethered ligand binding domains.

Some proteases can cleave PARs at several sites, including activation and disabling sites, and the net result depends on the efficiency of cleavage at different locations. For example, although cathepsin G can cleave PAR1 at the activation site Arg41-Ser42, the major cleavage site is at Phe55-Trp56, which removes the tethered ligand and disables the receptor (197). Although trypsin has been reported to activate PAR1, it also efficiently cleaves PAR1 at distal sites that would remove the tethered ligand domain. Indeed, in endothelial cells, trypsin inactivates PAR1, generating a receptor that is unresponsive to thrombin (200). In addition to cleavage at the activation site (Arg34-Ser35), trypase also cleaves PAR2 at the Lys41-Val42 site, which could inactivate the receptor (196). In the case of tryptase, the activating cleavage is more important since tryptase activates PAR2.

The consequences of exposing cells to proteases depends on the repertoire of PARs expressed by a cell and whether the proteases activate or disable particular receptors. For example, cathepsin G disables PAR1 but activates PAR4. Thus in human platelets, which express PAR1 and PAR4, cathepsin G can induce aggregation through PAR4 even though it disables PAR1 and thereby prevents signaling by low concentrations of thrombin (249). In contrast, in fibroblasts and endothelial cells that do not express PAR4, cathepsin G abolishes thrombin signaling through PAR1. In addition, proteolysis can impair the ability of PAR5 to act as a cofactor for PAR4 (74). In murine platelets, PAR3 binds thrombin but does not signal. Instead, it concentrates thrombin in the vicinity of PAR4 and thus serves as cofactor for PAR4 in response to low concentrations of thrombin. Cathepsin G does not cause aggregation of murine platelets but prevents aggregation to low concentrations of thrombin, indicating that cathepsin G abolishes this cofactor role of PAR3. It is very likely that proteases that destroy other nonreceptor cofactors, such as TF, would also have profound effects on PAR function.

B. PAR Signaling Is Attenuated by Receptor Desensitization

Proteases activate PARs by an irreversible mechanism: cleavage exposes the tethered ligand domain that is always available to interact with the cleaved receptor. Thus activation would result in prolonged signaling unless there were efficient mechanisms to attenuate the response. The principal mechanism that terminates signaling by PARs is broadly similar to the classical pathway of desensitization that has been described in detail for many other GPCRs, in particular rhodopsin and the β2-
adrenergic receptor (reviewed in Refs. 22, 181). The general mechanism of desensitization of these receptors is as follows (Fig. 6A). Ligand occupation of the GPCR induces the translocation of members of the family of G protein receptor kinases (GRKs) from the cytosol to the activated receptor at the cell surface. GRKs are serine-threonine kinases that phosphorylate activated GPCRs, usually within the carboxy terminus or third intracellular loop. Phosphorylation triggers the membrane translocation of arrestins, which interact with the phosphorylated GPCR, disrupt association with heterotrimeric G proteins, and thereby terminate signal transduction. Additional mechanisms may involve phosphorylation by second messenger kinases, which also terminate signaling. However, there remain many critical aspects of desensitization of GPCRs, including PARs, that are unexplored. In many cases, the GRK and arrestin isoforms that are coexpressed with receptors in question and which mediate desensitization to physiological stimuli are unknown. Most information about desensitization derives from studies of transfected receptors in cell lines; although genetically modified animals that are deficient in certain GRKs and arrestins are available, comparatively little is known about mechanisms of desensitization in vivo. Mechanisms of desensitization also vary between different PARs, probably due to structural differences, especially in the intracellular loop III and carboxy terminus.

1. PAR1

PAR1 activators trigger rapid phosphorylation of the receptor in transfected cell lines (136, 302, 304). Both GRKs and second messenger kinases could mediate this phosphorylation. Although activation of PKC stimulates PAR1 phosphorylation, thrombin-stimulated phosphorylation persists after inhibition or downregulation of PKC, suggesting other mechanisms of agonist-induced phosphorylation (136). One principal mediator of agonist-stimulated phosphorylation of PAR1 is GRK3, since overexpression of GRK3 enhances agonist-induced PAR1 phosphorylation and suppresses thrombin signaling, whereas GRK2 is considerably less effective. The importance of GRK3 in desensitization of PAR1 has been confirmed in vivo by observations in transgenic mice overexpressing GRK3 in the myocardium (133). Although signaling of the β2-adrenergic receptor is unaltered in these animals, since this receptor is mostly regulated by GRK2, thrombin-induced signaling is markedly attenuated by overexpression of GRK3. However, the observation that signaling of GRK3-insensitive mutants of PAR1 is still rapidly attenuated suggests the existence of additional mechanisms, perhaps involving other GRKs (136). GRK5 mediates PAR1 desensitization in endothelial cells, where GRK5 expression suppresses thrombin signaling and a dominant negative GRK5 mutant prolongs thrombin signals (278).

β-Arrestins are cofactors for GRKs; they interact with GRK-phosphorylated GPCRs at the cell surface and disrupt their association with heterotrimeric G proteins to terminate the signal. β-Arrestins also mediate desensitization of PAR1 (225). Desensitization of PAR1 is markedly diminished in embryonic fibroblasts prepared from mice lacking both β-arrestin-1 and -2, and also in fibroblasts lacking only β-arrestin-1, indicating a major role of β-arrestin-1 in desensitization of PAR1 in fibroblasts. This observation is the first to show a differential role of β-arrestin isoforms in desensitization of a GPCR.

Several observations suggest that modifications of the tethered ligand domain of cleaved PAR1 may also contribute to desensitization. One mechanism may involve cleavage of the tethered ligand domain. Exposure of platelets to PAR1 AP induces release of a PAR1 fragment by a process inhibited by soybean trypsin inhibitor, suggesting that a trypsin-like enzyme cleaves activated PAR1 (223). In rat astrocytes, thrombin exposure strongly desensitizes Ca2+ mobilization to a second exposure to thrombin and PAR1 AP (289). In contrast, exposure to PAR1 AP desensitizes the response to subsequent challenge with thrombin but not to AP. These findings led to the hypothesis that after activation with AP, the tethered ligand domain is proteolytically destroyed, rendering the receptor unresponsive to thrombin but not AP. Two observations support this hypothesis. First, as predicted, exposure to thermolysin, which removes the tethered ligand, generates a receptor that responds to PAR1 AP but not to thrombin. Second, treatment with soybean trypsin inhibitor enhances responses to thrombin and PAR1 AP, suggesting the existence of an exogenous protease that contributes to desensitization. The identity of the protease remains to be determined. The potential importance of modifications of the exposed tethered ligand is also illustrated by a study of PAR1 signaling in Sf9 insect cells. PAR1 signals are prolonged in these cells, even after washout of agonist (44). However, treatment with thermolysin, which removes the tethered ligand, attenuates the otherwise sustained Ca2+ response.

2. PAR2

Responses to PAR2 activators are rapidly attenuated and strongly desensitized to repeated exposure, indicating the existence of powerful mechanisms that control signaling, which have not been fully characterized. PKC appears to play an important role in regulating PAR2 (23, 86). Thus activation of PKC with phorbol esters abolishes PAR2-induced Ca2+ signaling in transfected cell lines and enterocytes that naturally express this receptor, whereas PKC inhibitors magnify responses to PAR2 activators. Mutation of a putative PKC site in the carboxy tail (ST336/6 → A) renders the receptor unresponsive to inhibition by phorbol esters (86). This receptor is also de-
sensitization defective: Ca$^{2+}$ responses to trypsin and PAR$\textsubscript{2}$ AP are exaggerated, and there is diminished desensitization to repeated stimulation. The role of GRKs and $\beta$-arrestins in PAR$\textsubscript{2}$ desensitization is unknown.

3. PAR$\textsubscript{3}$ and PAR$\textsubscript{4}$

Little is known about desensitization of PAR$\textsubscript{3}$ or PAR$\textsubscript{4}$. PAR$\textsubscript{3}$ has a very short carboxy tail, with possible implications for desensitization, since truncated receptors frequently exhibit diminished desensitization. PAR$\textsubscript{4}$ is not rapidly phosphorylated after activation, possibly due to lack of sites for GRK phosphorylation in the carboxy tail, and in consequence, PAR$\textsubscript{4}$ slowly desensitizes (263). The slow kinetics of desensitization may permit PAR$\textsubscript{4}$ to signal in a sustained fashion in human platelets.

C. PARs Are Downregulated by Intracellular Proteases

In addition to processes that regulate coupling of receptors to signaling pathways, cells also determine their responsiveness to agonists by regulating the levels of receptors that are expressed at the plasma membrane and which are thus accessible to agonists in the extracellular fluid. The level of expression of receptors at the cell surface is a balance between removal by endocytosis and replenishment by recycling or mobilization of intracellular pools. Many receptors internalize after binding agonists. However, the fate of endocytosed receptors depends on postendocytic sorting, which varies from receptor to receptor. At one extreme, some GPCRs are an ideal model to study these postendocytic sorting events since they are invariably targeted for degradation after activation. The molecular mechanisms and pathways of agonist-induced trafficking of PARs vary from receptor to receptor and in different cells.

1. PAR$\textsubscript{1}$

PAR$\textsubscript{1}$ is rapidly and extensively internalized after activation: stimuli trigger endocytosis of $>85\%$ PAR$\textsubscript{1}$ within 1 min in erythroleukemic and megakaryoblastic cell lines, although endocytosis proceeds a little more slowly in other cell types (129). The initial accumulation of PAR$\textsubscript{1}$ into coated pits suggests that internalization proceeds by a clathrin-mediated process. In support of this suggestion, activated PAR$\textsubscript{1}$ colocalizes with clathrin in PAR$\textsubscript{1}$-transfected fibroblasts, and disruption of clathrin with hypertonic sucrose (causes abnormal clathrin polymerization) and monodansylcadaverine (disrupts imaging of clathrin-coated pits) markedly inhibits endocytosis of activated PAR$\textsubscript{1}$ (281). The GTPase dynamin mediates detachment of clathrin-coated pits, and expression of a GTPase-defective dynamin mutant (K44E) also inhibits trafficking of activated PAR$\textsubscript{1}$. In addition to endocytosis of activated receptors, PAR$\textsubscript{1}$ also undergoes constitutive endocytosis in the absence of proteolytic activation. However, whereas activated PAR$\textsubscript{1}$ is mostly destined for lysosomal degradation (129), constitutively endocytosed PAR$\textsubscript{1}$ recycles to the plasma membrane (261). Indeed, there is a tonic endocytosis and recycling of PAR$\textsubscript{1}$ that maintains receptor at the cell surface and in an intracellular pool. Distinct domains exist in the carboxyl tail of PAR$\textsubscript{1}$ that are required for stimulated and constitutive endocytosis, suggesting that the two types are endocytosis proceeding by distinct mechanisms (262).

$\beta$-Arrestins play a major role in endocytosis of many GPCRs by serving as adaptor proteins that link GRK-phosphorylated receptors to clathrin and AP2. However, $\beta$-arrestins play no role in stimulated endocytosis of PAR$\textsubscript{1}$, which proceeds with normal kinetics in fibroblasts lacking $\beta$-arrestin-1 and -2, whereas agonist-induced endocytosis of the $\beta$-adrenergic receptor is inhibited in this system (225). In addition, constitutive PAR$\textsubscript{1}$ trafficking proceeds normally in fibroblasts that lack $\beta$-arrestins. Different adaptor proteins, such as AP2, may participate in endocytosis of PAR$\textsubscript{1}$.

Compared with our understanding of the mechanisms of endocytosis, less is known about the mechanisms of postendocytic sorting of internalized GPCRs, either to degradation or recycling pathways. Once internalized, most activated PAR$\textsubscript{1}$ is targeted to lysosomes for degradation. However, lysosomal sorting is not complete because in some cells there is considerable recycling (129). Progress has been made in defining the molecular mechanisms that target PAR$\textsubscript{1}$ to lysosomes. Sorting nexin-1 is a membrane-associated protein that interacts with the carboxy tail of the EGF receptor and is required for its downregulation. The yeast ortholog of sorting nexin-1, Vps5p, participates in targeting hydrolases to the vacuole, the yeast equivalent of the lysosome. In PAR$\textsubscript{1}$-transfected HeLa cells, agonist stimulation induces colocalization of PAR$\textsubscript{1}$ in endosomes with sorting nexin-1 (311). Expression of a carboxy-terminal deletion mutant of sorting nexin-1 has no effect on endocytosis of activated PAR$\textsubscript{1}$, but induces its accumulation in early...
endosomes and inhibits its degradation of lysosomes. Thus sorting nexin-1 participates in sorting of PAR₁ from early endosomes to lysosomes.

The domains of PAR₁ that specify targeting to lysosomes are not fully defined, although substitution of the carboxy tail of PAR₁ with the equivalent domain of the neurokinin-1 receptor (which recycles) alters PAR₁ trafficking to the recycling pathway (282, 283). This recycled receptor appears to be tonically active, which emphasizes the importance of lysosomal degradation as a mechanism that unequivocally terminates PAR₁ signaling. Conversely, the neurokinin-1 receptor with the carboxy tail of PAR₁ is targeted to lysosomes. Thus carboxy-terminal domains of PAR₁ are important for lysosomal trafficking. This lysosomal trafficking of PAR₁ (and PAR₂) proceeds whether the receptor is activated by proteolysis or by AP, and thus is not unique for the cleaved receptor.

2. PAR₂

Like PAR₁, activated PAR₂ is endocytosed and trafficked to lysosomes (23, 90). Endocytosis of PAR₂ proceeds by a clathrin-mediated mechanism since it is inhibited by blockade of clathrin function. Endocytosis of PAR₂ also requires dynamin, because GTPase defective dynamin K44E inhibits endocytosis (242). In contrast to PAR₁, endocytosis of PAR₂ requires β-arrestins. Thus activation of PAR₂ induces the marked and rapid translocation of β-arrestin-1 from the cytosol to the plasma membrane, followed by a prolonged colocalization of PAR₂ and β-arrestin-1 in early endosomes (86, 90). Eventually, PAR₂ and β-arrestin-1 are sorted into distinct compartments: PAR₂ is targeted to lysosomes, and β-arrestin-1 returns to the cytosol. The importance of β-arrestins in endocytosis of PAR₂ is indicated by two observations. First, expression of a dominant negative mutant of β-arrestin, β-arrestin³¹⁸⁻⁴¹⁹, which comprises the clathrin binding domain, prevents endocytosis. Second, a PAR₂ mutant, ST363/6 → A, fails to internalize and shows impaired desensitization, because of its inability to interact with β-arrestins. Thus PAR₂ appears to belong to the class B GPCRs that form high-affinity interactions with arrestins. β-Arrestins also play an important role in PAR₂-mediated activation of ERK1/2 as discussed in section μC (86).

Progress has been made in defining the mechanisms of intracellular trafficking of PAR₂. The rab GTPases mediate many steps of vesicular trafficking of proteins to and from the plasma membrane and between organelles. Rab5α colocalizes with PAR₂ in early endosomes within minutes of activation (242). Expression of a GTPase defective mutant, dominant negative rab5αS34N, disrupts early endosomes and inhibits endocytosis of activated PAR₂. Thus rab5α mediates distal steps in endocytic trafficking of PAR₂ from clathrin-coated pits to early endosomes. Once internalized, PAR₂ translocates to lysosomes and is degraded, as determined by Western blotting (Bunnett, unpublished observation). Although the molecular mechanisms that target PAR₂ for degradation are unknown, PAR₂ is extensively ubiquitinated after activation, determined by receptor immunoprecipitation and Western blotting for ubiquitin (Bunnett, unpublished observation), and ubiquitination of some other GPCRs is a prerequisite for degradation (185).

D. Sustained Signaling by Proteases Requires Mobilization and Synthesis of New Receptors

PARs are activated by an irreversible mechanism, and once cleaved, most activated PARs are destined for lysosomal degradation. Thus PARs are “one-shot” receptors, and sustained signaling requires the mobilization of intracellular stores of intact receptors or the synthesis of new receptors. The importance of these mechanisms depends on whether cells possess prominent intracellular stores of PAR₁. In megakaryoblasts, where most receptors are rapidly internalized after thrombin cleavage, recovery is a slow process that depends on the synthesis of new receptors (26, 129). In contrast, resensitization of responses to PAR₁ activators in endothelial cells and fibroblasts depends on mobilization of the prominent stores of PAR₁ in the Golgi apparatus (121, 125). Platelets, which lack both the ability to synthesize new receptors and prominent intracellular pools, are unable to repopulate the plasma membrane with new receptors after thrombin exposure and thus do not recover responsiveness to thrombin. Because platelets are only required to respond once to thrombin, by aggregation, the lack of a robust system of resensitization does not impair their function. Moreover, human platelets also express PAR₄, which facilitates prolonged signaling by thrombin.

Similar mechanisms account for the resensitization of PAR₂. There are prominent intracellular pools of PAR₂ in many cells, including enterocytes (23). Resensitization of responses to trypsin and the replenishment of the cell surface with intact PAR₂ is minimally affected by cycloheximide but attenuated by disruption of the pools with brefeldin A, suggesting that resensitization requires mobilization of pools. Similar mechanisms of PAR₂ resensitization have been observed in vascular tissues in vitro, suggesting that these are widespread mechanisms (113).

The role of rab GTPases in trafficking of PAR₂ from Golgi stores to the cell surface has been examined. Rab11a colocalizes in the Golgi apparatus with PAR₂, and PAR₂ activators stimulate redistribution of rab11a into vesicles containing PAR₂ that migrate to the cell surface (242). Expression of GTPase defective rab11aS25N causes retention of PAR₂ in the Golgi apparatus, and overexpression of wild-type rab11a accelerates both re-
covery of PAR2 at the cell surface and resensitization of PAR2 signaling. Thus rab11a plays an important role in PAR2 resensitization. Of interest, rab5a promotes and rab5aS34N impedes resensitization of cells to trypsin. Thus rab5a is required for PAR2 endocytosis and resensitization, whereas rab11a contributes to trafficking of PAR2 from the Golgi apparatus to the plasma membrane.

V. PROTEASE-ACTIVATED RECEPTORS: CONTRIBUTION TO PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL CONTROL MECHANISMS

Although much is known about the potential functions of PARs (Fig. 7), there remain substantial obstacles to understanding the role of proteases and PARs in physiology and disease. First, the system is complex: protease signaling depends on availability of activating or disabling proteases, protease inhibitors, and cofactors or anchoring proteins. Understanding the system requires knowledge of all of these components, and in many situations this information is lacking. Second, most studies rely on administration of activators (proteases or APs). Although such studies provide information about the potential role of PARs, they do not provide direct information about their function in physiology or diseases states. Proteases and certain APs are not selective activators of PARs, and APs are agonists at only very high concentrations (usually >1 μM). Peptides at high concentrations can have biological effects that are unrelated to receptor occupancy. For example, inactive analogs of PAR2 AP cause edema upon intraplantar injection (296). Third, selective antagonists of PARs are not widely available. Although antagonists have been developed for PAR1 (9), the intramolecular mechanism of activation of PARs may hamper the identification of antagonists by traditional methods involving screening of large chemical libraries. There are recent reports of peptide-based antagonists of PAR1 and PAR4.

![Diagram](http://physrev.physiology.org/)

**FIG. 7.** A summary of the potential physiological and pathophysiological roles of proteases and PARs in different cell types. Signaling depends on the availability of proteases, which can activate or disable PARs, protease inhibitors, PARs, and protease cofactors and anchoring proteins. Proteases signal to a wide variety of cells to regulate critically important biological processes, with implications for physiological regulation and disease.
(termed pepducins) that mimic intracellular domains of these receptors, but they have yet to be widely used (71, 72). Although it is generally more straightforward to develop drugs that inhibit proteases, it can be difficult to attain absolute selectivity between related enzymes.

In the absence of selective activators and antagonists, the best current approach to specifically determine the role of PARs in vivo is to study genetically modified animals. Mice have been developed that lack all known PARs (64, 83, 143, 177, 255, 313), and by selective crossing it will be possible to breed animals that lack several receptors. Transgenic animals have also been identified that overexpress PAR2 (255). However, the use of genetically modified mice is also fraught with problems. Effects of deletion on embryonic survival and fertility impede the breeding of animals for experimentation: approximately one-half of PAR1 knockout mice die before birth (64, 83). Compensatory alterations in the expression or activity of other PARs can occur in knockout animals (77), but in general these possibilities have not been thoroughly tested. There are also major differences in biological responses between different strains of mice, and it is essential to study wild-type and knockout animals in the identical genetic background, requiring the use of littermates or extensive back-crossing. Finally, many experimental models using mice are very imperfect models of human disease, requiring caution in extrapolating observations in mice to humans.

With these caveats in mind, we will briefly discuss the roles of proteases and their receptors in several systems.

A. Protease Signaling in the Circulatory and Cardiovascular Systems

The contributions of proteases and their receptors to regulation the cardiovascular system have been recently reviewed (50, 68, 226, 240).

1. Proteases

As discussed in section III, coagulation and anticoagulation factors, in particular thrombin, FVIIa, FXa, and APC, can regulate cells by cleaving PARs. Proteases released from inflammatory cells within the circulation and in tissues can also activate PARs in the circulatory and cardiovascular systems. For example, cathepsin G from neutrophils is a potential activator of PAR4 on platelets (249). Endothelial cells also express trypsins (169), which could activate PAR2 and PAR4, although almost nothing is known about the function of endothelial trypsins.

2. PARs

PARs are expressed by circulating cells as well as by endothelial and vascular smooth muscle cells. Platelets express several receptors for thrombin, although the combination varies between species: human platelets express PAR1 and PAR4 (142), murine platelets express PAR3 and PAR4 (143), and guinea pig platelets express PAR1, PAR3, and PAR4 (8). Neutrophils also express PAR2 (128). Human umbilical vein endothelial cells express PAR1, PAR2, and PAR3 (194, 218), and vascular smooth muscle cells express PAR1 and PAR2 (79).

3. PAR signaling to circulating cells

Platelets are circulating cells that are critical mediators of coagulation. Thrombin cleaves PARs on platelets to cause aggregation, granular secretion, and induction of procoagulant activity, which are essential for hemostasis. Thrombin signaling to platelets is enhanced by interaction with surface proteins such as glycoprotein Ibα (84) or by direct binding to PAR1 or PAR3, which possess hirudin-like thrombin-binding domains (135, 306). Thrombin signals by activating PAR1 and PAR4 in human platelets (142) and PAR4 alone in murine platelets, since PAR3 does not signal in murine platelets (199). What is the relative importance of PAR1 and PAR4 in human platelets? In isolated human platelets, antagonism of PAR1 blocks responses to low concentrations of thrombin, and antagonism of both receptors inhibits responses to low and high thrombin concentrations (142). Activation of PAR1 with AP is as efficacious as thrombin in inducing aggregation, Ca2+ influx, and development of procoagulant activity in human platelets, whereas PAR4 AP is less efficacious (7). Although these results could indicate that PAR1 is the primary thrombin receptor on human platelets, with PAR4 functioning in reserve, they may also reflect a generally lower efficacy of PAR4 AP as an agonist of that receptor. PAR1 may also contribute to sustained actions of thrombin on platelets and thereby mediate the late phase of platelet activation (70). In addition, PAR4 could act as a platelet receptor for other proteases, such as cathepsin G (249). In murine platelets, which express PAR3 and PAR4, PAR3 does not signal but instead anchors thrombin to the cell surface by its hirudin-like domain and thereby facilitates thrombin cleavage of PAR4 (199, see sect. uD2).

The contributions of PARs to coagulation have also been investigated in vivo by use of antagonists to PAR1. In cynomologus monkeys, whose platelets express PAR1 and PAR4, an antagonist of PAR1 inhibits thrombus formation and vessel occlusion in a model of electrolytic injury of the carotid artery (88). These studies indicate that PAR1 plays a predominant role in platelet regulation in this species. However, the importance of a dual receptor system on murine platelets in vivo is illustrated by the observation that deletion of PAR3 or PAR4 results in prolonged bleeding time and protection from ferric chloride-induced thrombosis of mesenteric arterioles and thromboplastin-induced pulmonary embolism (313). The
role of PAR4 in vivo has also been examined by use of novel antagonists, the pepducins (71). Peptides derived from the third intracellular loop of PAR1 and PAR4 with attached palmitate groups are potent inhibitors of thrombin-mediated aggregation of human platelets. Treatment of mice with a PAR4 pepducin prolongs bleeding time and protects against systemic platelet activation, consistent with the phenotype of PAR4-deficient mice (72). Thus PAR4 plays an important role in thrombin signaling in mice.

4. PAR signaling to the vessels

Proteases can signal to endothelial cells and vascular smooth muscle cells to control resistance and blood flow, extravasation of plasma proteins and granulocytes, and angiogenesis.

The effects of PAR activators on the contractile state of blood vessels has been extensively examined by classical organ bath pharmacology. The net effect of PAR activators depends on the receptor, the vessel, and whether the effect is mediated by release of agents from the endothelium of by direct actions on vascular smooth muscle. Thrombin and selective PAR1 agonists invariably cause relaxation of precontracted blood vessels, including large conduit-like vessels, for example, human pulmonary artery (116), porcine coronary artery (114), and rat aorta (184), and also of smaller resistance vessels, including human and porcine intramyocardial arteries (117). The consensus of these studies is that PAR1-induced relaxation is prevented by removal of the endothelium and is thus mediated by the secretion of a factor from endothelial cells that relaxes vascular smooth muscle. Relaxation is frequently suppressed by inhibition of nitric oxide (NO) synthase and is thus mediated in part by the release of NO from endothelial cells. However, there is also an NO-independent mechanism. In porcine coronary artery, this NO-independent mechanism involves the release of an endothelial-derived hyperpolarizing factor (114). In human pulmonary arteries, products of cyclooxygenase also contribute to relaxation (116). PAR1 activators can also contract certain vessels by endothelium-dependent and -independent mechanisms (277).

Activators of PAR2 also relax vascular tissues. Thus trypsin and PAR2 AP cause relaxation of a wide range of vessels from several species, including rat pulmonary artery (243) and human and porcine coronary and intramyocardial arteries (117). PAR2-mediated relaxation depends on the presence of the endothelium and involves both NO-dependent and -independent mechanisms. PAR2 AP also induces an endothelium-dependent contraction of, for example, rat aorta and renal, mesenteric, and pulmonary arteries (243). However, this contraction may involve a subtype of PAR2 in view of the different potencies with which analogs of the PAR2 AP induce contraction versus relaxation. The existence of a subtype receptor is also suggested by use of a “sandwich assay,” in which a segment of human umbilical vein is sandwiched next to a segment of endothelium-denuded rat aorta that serves as a “reporter” for the release of vasoactive agents from the human tissue (248). PAR2 AP induces release of an unknown factor from human umbilical vein that stimulates contraction of a rat aorta. Notably, trypsin does not have this effect, and the mechanism is thus independent of PAR2.

The effects of PAR activators on cardiovascular responses have been investigated in intact animals. In mice, intravenous injection of PAR1-selective AP causes a rapid and sustained hypotension, which is not NO mediated (47). Similarly, in rats and mice, PAR2 activators cause hypotension by NO-dependent and -independent mechanisms, which can be followed by a reflex hypertension (52). The effects of PAR4 activators on blood flow have recently been investigated in humans. Administration of PAR2 AP causes dilation of resistance vessels in the arm with an elevated blood flow, by NO- and prostaglandin-dependent mechanisms (241).

What are the physiological or pathophysiological implications of regulation of blood flow by PARs? Under normal circumstances it is unlikely that PARs regulate blood flow; indeed, there are no obvious abnormalities in the circulatory system in mice lacking PARs. However, PARs may play an important role during inflammation and sepsis, when there are elevated levels of proteases from inflammatory cells, the coagulation cascade and other sources, together with an elevated expression of PARs themselves. Exposure of endothelial cells in culture to inflammatory mediators such as TNF-α and lipopolysaccharide induces a selective upregulation of PAR2 but not PAR1 (216). Administration of endotoxin to rats also results in an upregulation of PAR2 in the vascular system that is accompanied by an increased hypotensive response to PAR2 activators (53). Similarly, exposure of segments of human coronary artery to interleukin-1α and TNF-α upregulates PAR2 and PAR4 and exacerbates PAR2- and PAR4-mediated relaxation of tissues (115).

Inflammation is accompanied by marked alterations in the permeability of blood vessels to plasma proteins and cells. Many inflammatory signals trigger the formation of gaps between endothelial cells of postcapillary venules, resulting in plasma extravasation and edema; these stimuli can also induce expression of adhesion molecules that promote the firm adhesion and ultimate infiltration of granulocytes into tissues. Proteases from the coagulation cascade and from inflammatory cells can trigger these events. The effects of thrombin and PAR1 on extravasation of plasma proteins have been studied in cultured endothelial cells and in the intact animal. Thrombin stimulates contraction of endothelial cells from the human umbilical vein, which results in gap formation and
The role of PAR1 in responses to mechanical damage is surprising that thrombin and PAR1 may play a role in view of the proangiogenic effects of thrombin, it is not clear if PAR2 activators on endothelial cells. Activation of PAR2 by the nervous system, but probably by direct effects of PAR4 since PAR4 AP also induces the response. In a similar manner, activation of PAR2 induces edema of the paw and also causes infiltration of granulocytes (298). In this case the edema is mediated in large part by the release of neuropeptides from sensory nerves and is thus dependent on a neurogenic mechanism (270). However, PAR2-induced infiltration of granulocytes is not mediated by the nervous system, but probably by direct effects of PAR2 activators on endothelial cells. Activation of PAR2 also stimulates leukocyte rolling, adhesion, and extravasation in postcapillary venules of the rat mesentery (297). However, PAR2-induced infiltration of granulocytes is not mediated by the nervous system, but probably by direct effects of PAR2 activators on endothelial cells. Activation of PAR2 mediated by the nervous system, but probably by direct effects of PAR2 activators on endothelial cells. Activation of PAR2 also stimulates leukocyte rolling, adhesion, and extravasation in postcapillary venules of the rat mesentery, by a mechanism that depends on release of platelet activating factor (294). Similarly, administration of PAR4 AP to the cremaster muscle of mice induces leukocyte rolling and adherence to venules (177). There is a modest decrease in the surgically induced rolling of neutrophils that is observed in PAR2-deficient mice, suggesting involvement of PAR2 in inflammation.

Activation of PAR1 by thrombin and AP stimulates proliferation of endothelial (194) and vascular smooth muscle cells (189). Activation of PAR1 similarly induces proliferation of vascular smooth muscle cells, suggesting that PAR1 and PAR4 may mediate the effects of thrombin (27). The proangiogenic effects of thrombin involve interactions with vascular endothelial growth factor (VEGF), itself a potent angiogenic factor. PAR1 activators upregulate expression of VEGF receptors (KDR and flt-1) by endothelial cells and potentiate the mitogenic effects of VEGF on endothelial cells (285). VEGF itself also promotes thrombin generation. These results suggest the existence of a mechanism of amplification of the angiogenic response involving PAR1 and VEGF receptors. In view of the proangiogenic effects of thrombin, it is not surprising that thrombin and PAR1 may play a role in vascular development. Deletion of prothrombin results in abnormalities of the vasculature of the yolk sac, and deletion of PAR1 results in partial embryonic lethality that is associated with bleeding abnormalities and which can be rescued by overexpression of PAR1 in endothelial cells (110). The role of PAR1 in responses to mechanical damage has also been evaluated in the mouse, which indicates involvement of PAR1 in regulation of the formation of the vascular matrix after injury (48). Activation of PAR2 also stimulates proliferation of endothelial cells (194). In a murine model of limb ischemia, trypsin and PAR2 AP promote angiogenesis and accelerate homodynamic recovery of the limb, suggesting a role for PAR2 in angiogenesis (191).

B. Protease Signaling to the Nervous System

The role of proteases and PARs in the nervous system is a topic of recent reviews (295, 308).

1. Proteases

Protease activators of neuronal PARs may derive from the circulation, from inflammatory cells that are resident in or recruited to neural tissues, or from neurons or astrocytes. Coagulation cascade proteases could signal to the nervous system during trauma and inflammation when there is elevated vascular permeability. However, these proteases are also expressed within the nervous system, although at low levels. Prothrombin transcripts are present in certain regions of the brain that also express PAR1 (91, 312). For example, in the olfactory bulb prothrombin and PAR1 mRNA are coexpressed in the mitral and granular cell layers. Elsewhere in the olfactory bulb and neocortex, the diffuse expression of prothrombin contrasts with the more defined expression of PAR1. Nevertheless, the potential exists for neuronally derived thrombin to activate PAR1 in the nervous system. Proteases from inflammatory cells may also signal to the nervous system. Granzyme A is released from CD4+ cytotoxic lymphocytes that infiltrate the brain during experimental allergic encephalomyelitis (272). Granzyme A cleaves a fragment of PAR1 at the activation site, and granzyme A signaling to astrocytes and neurons is inhibited by a PAR1 blocking antibody, suggesting that granzyme A activates PAR1. Neural tissues also express a large number of proteases that potentially activate PARs (252).

The biological effects of proteases are tempered by protease inhibitors, and neuronal tissues are replete with an array of inhibitors for proteases that activate PARs. The serpin protease nexin-1 is the most efficient endogenous inhibitor of thrombin. Nexin-1 mRNA is expressed in the brain where it is found in glial cells, synapses, and at high levels in capillaries and vascular smooth muscle in the human cortex (102). Thrombomodulin is a transmembrane glycoprotein that can form complexes with proteases and thereby regulates their activity at the cell surface. Thrombomodulin is expressed by astrocytes in culture, and expression of thrombomodulin is upregulated during trauma and consequent astrogliosis, as well as by activation of PAR2 (230). The upregulation of thrombomodulin by thrombin may thus serve to control activation of PAR1 on astrocytes.
2. PARs

Expression of PAR₁ mRNA in the rat brain is higher at birth compared with postnatal day 28, and levels increase again in adulthood (206, 207). PAR₁ is widely expressed throughout the rat brain, with higher levels of expression in particular regions, where it is likely to be present in neurochemically and functionally distinct cell types (312). Thus PAR₁ mRNA is expressed in the neocortex, cingulate/retrosplenial cortex, subiculum, nuclei within the hypothalamus and thalamus, and in discrete layers of the hippocampus, cerebellum, and olfactory bulb. In the hypothalamus, PAR₂ is present in dopaminergic neurons, but it is likely to be present in GABAergic granule cells in the olfactory bulb and in glutaminergic neurons of the anterior thalamus. PAR₂ is also expressed at low levels in glial cells throughout the brain, and by ependymal cells of the choroid plexus and ventricular lining where it could participate in control of transport of lining and in astrocytes from the rat brain (287, 288). The influx of extracellular Ca²⁺ in astrocytes depend on the concentration of activators and thereby participate in the control of cell shape and migration. Indeed, thrombin-stimulated neurite retraction and rounding of neurons is blocked by treatment of cells with Clostridium botulinum toxin C3 exoenzyme, which ADP-ribosylates and thereby inactivates small Rho GTP-binding proteins, implicating Rho in this action of thrombin and PAR₁ (138).

3. PAR signaling in the nervous system

The regulation of [Ca²⁺]ᵢ in neuronal cells is of fundamental importance in the control of excitability, release of neurotransmitters, and survival, and the signaling of proteases in neurons and astrocytes is commonly studied by measuring generation of second messengers, such as Ca²⁺. All four PARs are expressed in astrocytes (309). Thrombin and PAR₁ AP increase [Ca²⁺]ᵢ in a glioma cell line and in astrocytes from the rat brain (287, 288). The nature of the response depends on the duration of the stimulus: brief exposure triggers a rapid, transient increase in [Ca²⁺]ᵢ, whereas continued exposure induces oscillations or a sustained increase that are attributable to an influx of extracellular Ca²⁺. The influx of extracellular Ca²⁺ and the refilling of internal stores of Ca²⁺, which are both essential for oscillations in [Ca²⁺]ᵢ, depend on activation of protein kinase C by PAR₁. The response of astrocytes to thrombin may be mediated by two co-expressed receptors, PAR₁ and PAR₄ (148). Thus, in primary cultures of human astrocytomas, thrombin as well as PAR₁ and PAR₄ AP induce elevations in [Ca²⁺]ᵢ. Normal brain astrocytes express PAR₂ and respond to trypsin and PAR₂ AP (290). Selective agonists of PAR₁ and PAR₂ also signal enteric neurons and primary spinal afferent neurons to increase [Ca²⁺]ᵢ, providing functional evidence for expression of these receptors (67, 87, 270).

4. Morphology

Innervation depends on the control of neurite outgrowth, and there is great interest in understanding the factors that control this process. Proteases and protease inhibitors regulate the outgrowth of neurites. Neuroblastoma cells and certain primary cultures of neurons rapidly extend neurites when switched from serum-containing to serum-free culture medium. Thrombin inhibits neurite sprouting in serum-free medium (112). Conversely, neurite outgrowth in the presence of serum is stimulated by the thrombin inhibitors protease nexin-1 and hirudin, probably due to inhibition of thrombin in serum. Thrombin similarly inhibits the outgrowth of neurites from dorsal root ganglia (106). Astrocytes contribute to the blood-brain barrier, and there is considerable interest in understanding the control of astrocytes morphology. Protease and protease inhibitors regulate the morphology of astrocytes (14, 39). When cultured in serum, astrocytes adopt a flattened, fibroblast-like morphology, whereas in serum-free conditions they become stellate and project long processes. Thrombin mimics the effects of serum, whereas protease nexin-1 reverses the effect of thrombin. These effects of thrombin on the morphology of neurons and astrocytes are mimicked by PAR₁ AP and are thus likely attributable to activation of PAR₁. Rho GTPases regulate the actin-based cytoskeleton and thereby participate in the control of cell shape and migration. Indeed, thrombin-stimulated neurite retraction and rounding of neurons is blocked by treatment of cells with Clostridium botulinum toxin C3 exoenzyme, which ADP-ribosylates and thereby inactivates small Rho GTP-binding proteins, implicating Rho in this action of thrombin and PAR₁ (138).
thalamic neurons from death in response to environmental insults, including hypoglycemia and oxidative stress (293). Similarly, thrombin (50 pM, 1 h) and PAR1 AP protect hippocampal neurons in brain slices from death in response to experimental ischemia (271). This protective effect of thrombin is also observed in vivo. Thus brief occlusion of the common carotid arteries of gerbils induces tolerance of hypothalamic CA1 neurons to a more sustained ischemia. Intracerebral injection of the thrombin inhibitor hirudin prevents this protection, indicating involvement of thrombin (271). Thrombin-induced protection of astrocytes from hypoglycemia is attenuated by general inhibitors of serine/threonine kinases and by inhibition of Rho, suggesting involvement of the actin cytoskeleton (92). Another protease that activates PAR1, APC, also protects the brain from ischemic injury by a PAR1-dependent mechanism. Thus the protective actions of APC in a model of ischemic brain injury in mice are markedly attenuated by administration of a PAR1 blocking antibody (46).

However, activation of PAR1 also induce neurodegeneration. Prolonged exposure of neurons and astrocytes to high concentrations of thrombin (500–750 nM) causes apoptosis (93), and thrombin (100 nM, 1 h) decreases survival of hippocampal neurons in brain slices after hypoglycemic and hypoxic stress (271). The same kinase and Rho-dependent pathways that mediate protection also induce apoptosis, the difference resting on the duration and extent of receptor activation. Moreover, whereas low concentrations of thrombin induce transient elevations in \([\text{Ca}^{2+}]_i\) in hippocampal neurons, high concentrations induce large and sustained elevations, which may well trigger apoptosis (271). Supporting this concept, caspase-3 inhibitors prevent PAR1-induced death of motoneurons in the avian embryo (286). Some of the detrimental effects of thrombin and PAR1 APs could be mediated by the N-methyl-D-aspartate (NMDA) receptor system, which contributes to degeneration in the nervous system. Thus PAR1 activation potentiates NMDA currents in hippocampal neurons, raising the possibility that thrombin could exacerbate glutamate-induced neurotoxicity (107). In addition to effects on survival, activation of PAR1 also induces proliferation of astrocytes (229). The dual protective and degenerative effects of PAR1 could have implications for survival of neurons after injury: immediately after trauma and generation of thrombin, PAR1 may serve to protect the brain from damage; thereafter, PAR1 may have deleterious effects.

PAR4 may also contribute to inflammation in the brain, with detrimental effects (274). Thus PAR4 AP, but not PAR1 AP, stimulates release of TNF-\(\alpha\) from brain microglia, an effect that is PAR4 specific because it is prevented by downregulation of PAR4 with antisense. PAR4 AP also activates the inflammatory NF\(\kappa\)B pathway in these cells. Thus antagonists of PAR4 could be effective therapies for neurotrauma.

7. Neurogenic inflammation and pain

A subpopulation of primary sensory neurons with cell bodies in dorsal root, trigeminal, and vagal (nodose and jugular) ganglia express the neuropeptides SP and CGRP. These neurons have small, dark cell bodies with unmyelinated (C) or thinly myelinated (A-\(\delta\)) fibers and participate in neurogenic inflammation and nociception. The peripheral projections of these fibers detect thermal, chemical, and high-threshold mechanical stimuli in many tissues, including the skin, gastrointestinal tract, and airway. Stimulation results in the release of SP and CGRP from endings of primary sensory neurons in peripheral tissues, which initiates a variety of responses, collectively referred to as “neurogenic inflammation,” and characterized by arteriolar vasodilatation, extravasation of plasma proteins from postcapillary venules, and adhesion of leucocytes to the venular endothelium. These same stimuli also generate action potentials that are transmitted centrally where they induce the release of neuropeptides within the central nervous system, resulting in the central transmission of nociceptive signals. Proteases that activate PAR1 and PAR4 can signal to these neurons in peripheral and perhaps central tissues to control inflammation and pain (11, 87, 270, 296) (Fig. 8).

The role of PAR4 in sensory neurons has been extensively investigated. PAR4 is expressed by \(\sim\)65% of neurons in the dorsal root ganglia of the rat; 25–35% of these PAR4-expressing neurons also express SP and CGRP, respectively, and are thus involved in inflammation and pain (270). Trypsin, trypsin, and PAR4 AP signal to these neurons to elevate \([\text{Ca}^{2+}]_i\) and to stimulate secretion of SP and CGRP from segments of the dorsal horn, bladder, and atra, indicating that activation of PAR4 triggers peptide release from both the central and peripheral projections of these neurons. The intraplantar injection of PAR4 activators causes a sustained (>6 h) edema that is mediated by release of SP and CGRP from the peripheral projections of sensory nerves. Activation of PAR2 also induces hyperalgesia (149, 150, 296). Thus intraplantar injection of subinflammatory doses of PAR2 AP causes sustained (>24 h) mechanical and thermal hyperalgesia, with increased expression of fos protein in spinal neurons, indicative of neuronal activation. Hyperalgesia is not observed in mice lacking the neurokinin-1 (SP) receptor or preprotachykinin A, which encodes SP and neurokinin A. Neurokinin-1 receptor antagonists also prevent PAR2-induced hyperalgesia, but they are effective only if capable of penetrating the spinal cord or if delivered intrathecally. Thus PAR2-mediated hyperalgesia depends on the release of SP from the central projections of primary spinal afferent neurons. Together, these findings
suggest that proteases that activate PAR2 can signal to the peripheral projections of primary spinal afferent neurons to trigger release of neuropeptides from their peripheral and central projections; peripheral release causes neurogenic inflammation, and central release induces thermal hyperalgesia. The identity of the PAR2 activating proteases that mediate these effects remains to be determined, but one possibility is tryptase from mast cells. Mast cells containing tryptase are in close proximity to sensory nerve endings in uninflamed and inflamed tissues (267). Indeed, PAR2-deficient mice show diminished thermal hyperalgesia after intraplantar injection of compound 48/80 to degranulate mast cells, suggesting a role for PAR2 in mast cell-dependent hyperalgesia (296). PAR2 activators also induce pain in the pancreas by a neurogenic mechanism that involves activation of pancreatic sensory nerves (124). This observation is relevant to the painful condition of pancreatitis, where trypsinogen is prematurely activated within the pancreas and could cause hyperalgesia by activating PAR2 on pancreatic neurons.

The molecular mechanism of PAR2-mediated inflammation and hyperalgesia is unknown. However, it is likely to involve signaling events that regulate activity or expression of ion channels. One candidate is vanilloid receptor-1 (VR1), a member of the transient receptor potential (TRP) family of channels. TRPV1 is a nonselective cation channel that is activated by protons, elevated temperature, and certain lipids, as well as by exogenous vanilloids such as capsaicin (37, 141). TRPV1 plays an important role in inflammatory pain and thermal hyperalgesia, and several inflammatory agents transactivate this channel. The TRPV1 antagonist capsaicin antagonizes PAR2-mediated thermal hyperalgesia (152). Moreover, activation of PAR2 enhances TRPV1-induced Ca2+ signaling and TRPV1 currents in cell lines and neurons by a PKC-dependent mechanism (Bunnett, unpublished observation) and also potentiates capsaicin-induced release of CGRP (124). These results suggest that activated PAR2 couples PKC, which may phosphorylate and activate TRPV1, thereby inducing hyperalgesia.

In a similar manner, activation of PAR1 promotes extravasation of plasma proteins in many tissues by a mechanism that depends on the release of SP from the peripheral projections of afferent neurons (87). However, in contrast to PAR2, PAR1 activation causes somatic analgesia, by mechanisms that remain to be defined (11, 151).

C. Protease Signaling in the Gastrointestinal System

All PARs are expressed in the gastrointestinal tract, although most studies have focused on PAR2, which appears to be of particular importance in the gut. The role of PARs in the gastrointestinal tract has been recently reviewed (295).

1. Proteases

Of all organ systems, the gastrointestinal tract is exposed to the widest array of proteases under physi-
logical circumstances and during diseases. Digestive pro-
teases from glands in the stomach and intestine as well as
from extrinsic sources such as the pancreas bathe the
lumen of the gastrointestinal tract during and after feed-
ing. Thus physiological concentrations of trypsin in the
lumen of the small intestine can signal to enterocytes by
cleaving PAR2 at the apical membrane (167). The intesti-
nal lumen is also replete with bacteria and thus exposed
to bacterial proteases. Under normal circumstances, the
tight junctions of epithelial cells act as a barrier to prevent
the ingress of proteases from the lumen into tissues.
However, during stress and in the ingress of proteases from the lumen into tissues.

The effects of PAR activation on electrolyte secretion
by cultured enterocytes and sheets of mucosa have been
examined using Ussing chambers, allowing application of
agonists to apical (lumen) and basolateral (serosal) sur-
face and measurement of short-circuit current as an index
of ion secretion. The role of PAR1 in electrolyte secretion
has been examined using SCBN cells, a nontransformed
epithelial cell line from the crypts of the human small
intestine (30, 31). Application of thrombin or a selective
PAR1 AP to the basolateral surface of these cells stimu-
lates an increase in short-circuit current that is attribut-
able to Cl\textsuperscript{−} secretion by a Ca\textsuperscript{2+}-dependent mechanism.
Although SCBN cells also express the cystic fibrosis trans-
membrane conductance regulator (CFTR), activation of
PAR1 does not raise intracellular cAMP, and thus PAR1 is
unlikely to regulate the CFTR. PAR1 agonists induce ac-
tivation of Src, transactivation of the EGF receptor, activ-
ation of ERK1/2, phosphorylation of cytoplasmic phos-
pholipase A\textsubscript{2}, and stimulation of cyclooxygenase-1 and -2,
which induce Cl\textsuperscript{−} secretion.

PAR2 signaling in enterocytes has been investigated
using hBRIE cells, a nontransformed epithelial cell line
from the rat small intestine that retains many character-
istics of enterocytes. Trypsin and PAR2 AP stimulate gen-
eration of InsP\textsubscript{3}, mobilization of Ca\textsuperscript{2+}, and release of
arachidonic acid and prostaglandins E\textsubscript{2} and F\textsubscript{1\alpha} (167). The
generation of prostaglandins is also stimulated by the
application of physiological concentrations of trypsin to
the apical membrane of everted sacs of rat jejunum, sug-
uggesting expression of PAR2 at the apical membrane. Pro-
staglandins released from enterocytes may have a protec-
tive function and could also regulate fluid and electrolyte
secretion. Application of PAR2 AP and activating pro-
teases to the serosal surface of segments of intestine from
rat (300), mouse (73), and pig (109) induces an increase in
short-circuit current due to stimulation of Cl\textsuperscript{−} secretion,
but the mechanisms vary between species and perhaps
between different regions of the intestine. In the rat jeju-
num, Cl\textsuperscript{−} secretion depends on generation of prostaglan-
dins (300). However, in view of the very different poten-
cies of PAR2 agonists in this preparation and a PAR2\textsuperscript{−}
transfected cell line, Cl\textsuperscript{−} secretion may be due to the
activation of a variant of PAR2. In the pig ileum, PAR2\textsuperscript{−}
stimulated Cl\textsuperscript{−} secretion depends both on eicosanoids
and on submucosal neurons, since the stimulation is
strongly suppressed by the neuronal toxin saxitoxin
(109). Thus application of PAR2 agonists to the serosa
may activate submucosal neurons, which then release
stimulants of Cl\textsuperscript{−} secretion. In the distal colon of the
mouse, application of PAR2 agonists to the serosal sur-
face stimulates Cl\textsuperscript{−} and K\textsuperscript{+} secretion and inhibits amilio-
ride-sensitive Na\textsuperscript{+} absorption (73). These effects are me-
diated in part by the enteric nervous system.

PAR2 is also expressed by epithelial cells of the pan-
creatic duct, where it regulates ion transport. Trypsin and
PAR2 AP stimulate short-circuit current when added to the basolateral but not apical surface of monolayers of duct cells from the canine pancreas by stimulating Ca\(^{2+}\)-activated Cl\(^{-}\) channels and K\(^{+}\) channels (205). In this manner PAR2 may play a protective role in pancreatitis, when there is premature activation of trypsinogen and secretion of trypsin into the interstitial fluid. Activation of PAR2 on the basolateral membrane of duct cells could increase ductal secretion and promote clearance of toxins and debris.

4. Motility

Activation of PAR\(_1\) and PAR\(_2\) stimulates contraction of gastric longitudinal muscle from rats and guinea pigs, which is blocked by the cyclooxygenase inhibitor indo- methacin and the tyrosine kinase inhibitor genistein (3, 247). In the mouse gastric fundus, activation of PAR\(_1\) and PAR\(_2\) has a biphasic effect, causing relaxation and then contraction (59). The relaxation is blocked by apamin or ryanodine, indicating that it is mediated by ryanodine-sensitive and -insensitive activation of small-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels. In the longitudinal muscle of the rat colon, PAR\(_2\) APs inhibit spontaneous contractions (66). PAR\(_1\) and PAR\(_2\) APs also inhibit contraction of colonic circular muscle, which is prevented by apamin (198). Some muscles express more than one receptor for the same protease, and the net effect on motility depends on which receptor is activated. In the rat esophagus, PAR\(_1\) APs stimulate contraction, but PAR\(_4\) APs have little effect (154). However, in carbachol-precontracted tissues, PAR\(_1\) agonists cause additional contraction, whereas PAR\(_4\) agonists induce a strong relaxation. These results suggest that thrombin could have a dual effect in the esophagus, causing contraction by PAR\(_1\) and relaxation by PAR\(_4\). The dominant effect would depend on the concentration of thrombin: low concentrations preferentially activate PAR\(_1\), but high concentrations of thrombin are needed to activate PAR\(_4\). The intraperitoneal administration of selective APs of PAR\(_1\) and PAR\(_2\) to mice accelerates intestinal transit (155). These effects are abolished by verapamil, an inhibitor of the L-type Ca\(^{2+}\) channel, but are potentiated by apamin. Thus the net effects of PAR\(_1\) and PAR\(_2\) activators in vivo are to promote intestinal transit.

5. Exocrine secretion

PAR\(_2\) regulates pancreatic, salivary, and gastric secretions. PAR\(_2\) activation stimulates the release of amylase from isolated rat pancreatic acini (24). Intravenous injection of PAR\(_2\) AP in rats and mice also stimulates pancreatic amylase secretion, and the effect in mice is mediated by NO (158). PAR\(_2\) is also present in acini of the salivary gland, and PAR\(_2\) APs stimulate release of amylase and mucus in rats and mice (158). In mice, the effect on amylase secretion is partially mediated by NO, and the stimulation of mucin secretion in rats depends on activation of tyrosine kinase (157). Activation of PAR\(_2\) stimulates secretion of gastric, but not duodenal, mucus (153). In this manner, PAR\(_2\) APs protect the gastric mucosa from injury induced by acid-ethanol or by indomethacin. PAR\(_2\)-induced mucus secretion and gastric protection are suppressed by antagonists of the CGRP and neurokinin-2 receptors and by the TRPV1 antagonist capsazepine, and thus depend on the release of neuropeptides from sensory nerves (152). In contrast, PAR\(_2\)-mediated salivary secretion is independent of capsaicin-sensitive sensory neurons (156), an observation that illustrates the complexity of the PAR\(_2\) signaling system, which varies between tissues. PAR\(_2\) is also expressed by the pepsin-secreting chief cells of the stomach, and PAR\(_2\) agonists stimulate pepsin secretion in rats (160). Conversely, activation of PAR\(_2\) suppresses gastric acid secretion in intact animals (211). Gastric epithelial cells also express PAR\(_1\), and PAR\(_1\) AP stimulates secretion of mucus and prostaglandin E\(_2\), which may be protective (279).

6. Neurotransmission

The enteric nervous system comprises a series of ganglionated nerve plexuses that comprise the intrinsic nervous system of the gut. These ganglia include primary afferent neurons, interneurons, and motor neurons, allowing local, reflex regulation of all aspects of gastrointestinal function, including control of motility, exocrine and endocrine secretion, and fluid and electrolyte transport. The expression of PARs in neurons of the myenteric plexus suggests a role for proteases in control of motility. Indeed, PAR\(_1\), PAR\(_2\), and PAR\(_4\) are expressed by a substantial proportion of myenteric neurons in the guinea pig intestine (>50% for PAR\(_1\), PAR\(_2\)) (67, 104, 176). Thrombin, trypsin, and tryptase induce a slow and prolonged depolarization in myenteric neurons that is often accompanied by increased excitability (104, 176). Selective APs for PAR\(_1\), PAR\(_2\), and PAR\(_4\) similarly excite these neurons. Neurons of the enteric nervous system can be classified on the basis of their electrophysiological behavior. Two prominent types of neurons are AH, which are the intrinsic primary afferent neurons, and S neurons, which are motor neurons and interneurons. Approximately 50–60% of AH neurons and slightly fewer S neurons respond to PAR\(_2\) agonists. The PAR\(_2\)-responsive S neurons also express NO synthase and project axons in an anal direction. These are inhibitory motor neurons, and activation would be expected to suppress motility. PAR\(_2\) is also expressed by neurons of the submucosal nerve plexus of the guinea pig small intestine, some of which also express vasoactive intestinal polypeptide and are thus secretomotor neurons that play an important role in the control of fluid and electrolyte transport (235). Tryptsin, trypatase, and PAR\(_2\) AP evoke transient depolarization of submucosal neurons.
followed by a prolonged hyperexcitability that can last for several hours. This remarkable long-term hyperexcitability mimics that observed after degranulation of mast cells in the presence of antagonists of established excitatory mast cell mediators (histamine, serotonin, prostaglandins). Mast cell degranulation results in release of proteases, which desensitizes neurons to other activators of PAR2. Thus PAR2 AP and proteases from degranulated mast cells induce long-term excitability of submucosal neurons, which could result in enhanced fluid and electrolyte secretion from the mucosa (73, 109). PAR2 is also expressed by extrinsic neurons that innervate the intestine. Thus the administration of PAR2 activators into the lumen of the rat intestine induces a hyperalgesia to distension of the intestine with a balloon, consistent with a visceral hyperalgesia (60). However, in contrast to the somatic hyperalgesia that depends on the spinal release of SP, PAR2-dependent visceral hyperalgesia involves peripheral release of SP. The widespread expression of PARs in the intrinsic and extrinsic nervous systems of the gut may be of considerable interest during inflammation, where proteases from the circulation, inflammatory cells, and the intestinal lumen could alter neuronal excitability and induce alterations in motility, secretion, and pain perception.

7. Intestinal inflammation

Since intestinal inflammation is associated with the generation and release of proteases that are potential activators of PARs, there has been considerable interest in the role of PARs in inflammatory diseases of the intestine. Administration of the trypsin, tryptase, and PAR2 AP into the lumen of the mouse colon provokes a generalized inflammatory reaction in wild-type but not PAR2-deficient mice (40, 41). PAR2-induced inflammation is suppressed by inhibition of NO synthase, by ablation of sensory nerves, and by antagonism of the CGRP type 1 receptor and the neurokinin-1 receptor. Thus proteases induce inflammation in part by a neurogenic mechanism that involves the release of peptides from sensory nerves. Intracolonic PAR2 activators also disrupt the intestinal barrier integrity as observed by the increased paracellular permeability to bacteria. This disruption of tight junctions depends on activation of myosin light-chain kinase (41). Given the large presence and increased activity in the intestinal lumen of trypsin and tryptase, which can activate PAR2, these data have important implications for the pathophysiology of inflammatory bowel diseases. However, PAR2 may also serve a protective function in the intestine. Activation of PAR2 induces release of prostaglandins from enterocytes (167) and stimulates gastric mucus secretion (153), both of which are protective. Administration of PAR2 AP in mice protects against inflammation induced by the rectal administration of dinitrobenzene sulfonic acid, a widely used model of hapten-induced colitis (103). This protective effect is also neurogenic, since it is prevented by ablation of sensory nerves and antagonism of the CGRP 1 receptor.

D. Protease Signaling in the Airway

The potential involvement of PARs in regulation of the airways and in pulmonary disease has been recently reviewed (58, 127).

1. Proteases

During inflammation and trauma, proteases from the coagulation cascade and from inflammatory cells could regulate pulmonary cells by activating PARs. Indeed, there are elevated levels of coagulation proteases (134) and tryptase (139) in bronchoalveolar lavage from patients with chronic inflammatory diseases. Proteases that activate PARs may also arise from cells in the airways. Immunoreactive trypsinogen colocalizes with PAR2 in Clara cells of the epithelium of human bronchioles, whereas in larger bronchioles trypsinogen is expressed by cells just above the basal layer (56, 58). In addition, a trypsinlike enzyme can be detected by zymography in the bronchoalveolar lavage collected from mice treated intranasally with the bacterial endotoxin lipopolysaccharide. Intriguingly, certain nonmammalian proteases such as dust mite allergens can also signal to cells in the airway by cleaving PARs, which may have implications for allergic inflammation (273).

2. PARs

The PARs are expressed by many cell types in the airway. There is weak staining for immunoreactive PAR1 in ciliated and basal epithelial cells and in smooth muscle cells of rat trachea and bronchi (49, 127). In rat, mouse, guinea pig, and human, immunoreactive PAR2 is expressed by ciliated and nonciliated epithelial cells, especially at the apical membrane, as well as in glands, airway smooth muscle, and endothelial cells and vascular smooth muscle cells (49, 56, 58, 79, 192, 237, 255).

3. Airway resistance

Given the importance of increased airway resistance in asthma, there is great interest in understanding the regulation of contraction of airway smooth muscle. There is evidence that PAR2 activators induce both bronchodilatation, which would be protective, and detrimental bronchoconstriction.

The first evidence in favor of a protective role came from the finding that activation of PAR2 induces relaxation of isolated bronchi from several species, including mouse, rat, guinea pig, and human, precontracted with...
carbachol (49, 56, 58, 172). This relaxation requires the presence of the epithelium and is mediated by prostanoids but not by NO. Surprisingly, in the rat, PAR₂ AP but not trypsin causes relaxation, possibly due to the existence of trypsin inhibitors in that tissue (49). PAR₂-induced relaxation of the isolated mouse trachea is unaffected by acute damage to the epithelium caused either by mechanical rubbing or infection with influenza A virus (173). However, the effect of PAR₂ activation in the airway may depend on the tissue chosen for study and the species. For example, whereas PAR₂ activation causes relaxation of isolated guinea pig trachea and main bronchi by a mechanism that is dependent on the presence of the epithelium and that is mediated by NO and prostanoids, in smaller intrapulmonary bronchi the sole effect of trypsin and PAR₂ AP is to trigger smooth muscle contraction (237). PAR₂ activators also stimulate contraction of lobar or segmental bronchial rings from humans that is mediated in part by a direct activation of PAR₂ on smooth muscle cells (256). Since smaller bronchioles are the principal site of airway resistance, contraction would be expected to increase total airway resistance, which would be detrimental. In addition to the aforementioned indirect effects of PAR₂, which often require the presence of the epithelium, PAR₂ activators, including trypsin, can directly signal to isolated smooth muscle cells from the human airway by mobilizing intracellular Ca²⁺ (19, 256).

Studies in intact animals also show a protective or detrimental effect of PAR₂ activation. In support of a protective role, aerosol administration of PAR₂ AP to intact rats inhibits serotonin-induced bronchoconstriction (56, 58). Similarly, intravenous administration of PAR₂ AP inhibits histamine-induced increase in airway resistance in guinea pigs by a mechanism independent of the release of prostaglandins, NO, or the effect of circulating epinephrine (51). In contrast, intravenous and intratracheal administration PAR₂ activators causes bronchoconstriction in intact guinea pigs, mediated in part by prostanoids and involving the release of tachykinins from sensory nerve endings and activation of neurokinin-1 and -2 receptors (237). Thus PAR₂ may be protective or detrimental in the airways, depending on the experimental model and species. It will be of great interest to examine the contribution of proteases and PAR₂ to airway disease by using specific antagonists and genetically modified animals.

4. Electrolyte transport

PAR₂ also regulates ion transport in the airway epithelium (78, 171). PAR₂ activators, added to the basolateral surface of bronchial epithelial cells and airway tissue, cause a transient Ca²⁺-dependent increase in short-circuit current, followed by a sustained inhibition of amiloride-sensitive current. This mechanism may control fluid volume and composition at the airway surface.

5. Inflammation and fibrosis

There has been considerable interest in the role of tryptase in airway inflammation and asthma. Hyperplasia of smooth muscle contributes to remodeling of the airways that accompanies asthma, and tryptase induces proliferation of airway smooth muscle cells, fibroblasts, and epithelial cells (29, 33, 244). Tryptase also induces hyperresponsiveness in the airway (17), and when injected into the skin tryptase causes plasma extravasation and neutrophil infiltration (119, 120). In a sheep model, inhaled tryptase causes bronchial hyperresponsiveness, whereas a tryptase inhibitor reduces early and late phase bronchoconstriction and hyperresponsiveness to allergen challenge (55). Although it is not established that these effects of tryptase are mediated by PAR₂, PAR₂ AP does have similar effects. For example, PAR₂ AP also induces proliferation of airway smooth muscle cells and lung fibroblasts, and thus PAR₂ may mediate the proliferation effects of tryptase on lung fibroblasts and myocytes (2, 18).

PAR₂ may have both proinflammatory and protective effects in the airway. Airway epithelial cells express PAR₁, PAR₂, and PAR₄, and activation of these receptors triggers the release of cytokines such as interleukin-6, interleukin-8, and prostaglandin E₂ that can regulate inflammation (12). Observations on PAR₂ knockout mice and animals overexpressing human PAR₂ suggest that this receptor contributes to allergic inflammation (255). In an ovalbumin-induced allergic inflammation of the airway, both the infiltration of eosinophils into the airway lumen and methacholine-induced airway hyperreactivity are markedly diminished in PAR₂-deficient animals, and exacerbated in mice overexpressing PAR₂. PAR₂ deletion also reduces IgE levels to ovalbumin sensitization by fourfold compared with wild-type animals. These results, together with the observation of upregulation of PAR₂ in airway epithelium of asthmatics (164), suggest that PAR₂ contributes to the development of immunity and to allergic inflammation of the airway. However, PAR₂ can also be protective against airway inflammation. Intranasal delivery of PAR₂ AP in mice does not cause any inflammation in the airways but inhibits the marked immune cell inflammatory response, characterized by a recruitment of polymorphonuclear leukocytes, triggered by administration of lipopolysaccharide (195).

Although less is known about PAR₁ in the airway, there are reports of a role for thrombin and PAR₁ in fibrotic diseases. Thrombin and PAR₁ AP are mitogenic for human airway smooth muscle cells (280). Thrombin also stimulates the release of the proinflammatory and fibrogenic cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) from airway smooth muscle...
PAR2 immunoreactivity is predominantly localized to the
basal and suprabasal spinous layers, although the stratum
cornueum is unstained (76). PAR2 is also expressed in the
inner root sheath of hair follicles and in myoepithelial
cells of sweat glands. Keratinocytes in culture also ex-
press PAR2 mRNA and protein (251). PAR2 mRNA is also
moderately expressed in cultured human microvascular-
ture endothelial cells, but melanocytes do not express
PAR2 (126, 268). The expression of PAR2 has been eval-
uated in some skin diseases (268). Thus PAR2 immuno-
activity is strong in the hyperproliferative granular layer
of skin from patients with lichen planus, and in atopic
dermatitis there is strong staining of the whole epidermis,
as well as in blood vessels and dendritic-like cells.

3. Pigmentation

There is considerable interest in the role of PAR2 in
pigmentation. The interaction between keratinocytes and
neighboring melanocytes is essential for skin pigmen-
tation. Melanocytes produce secretory granules, melan-
osomes, which produce melanin. Melanin-containing me-
lanosomes are transferred to dendrites of melanocytes and
are then taken up by keratinocytes by phagocytosis, re-
sulting in skin darkening. Although melanocytes do not
express PAR2, PAR2 activators regulate the phagocytosis
of melanosomes by keratinocytes and thereby control
pigmentation (259, 264). Thus trypsin and PAR2 AP stim-
ulate pigmentation of cocultures of keratinocytes and
melanocytes. Remarkably, the topical application of PAR2
AP induces pigmentation of human skin transplanted
ontto mice. Conversely, a serine protease inhibitor causes
lightening of the skin (224). The mechanism of these
effects of PAR2 on pigmentation depends on the stimula-
tion of phagocytosis in keratinocytes. Thus activation of
PAR2 increases the ingestion of particles and bacteria by
keratinocytes (264). Phagocytosis by keratinocytes not
only plays a role in pigmentation but also enables “non-
immune” cells such as these to participate in protection of
the skin, with implications for wound healing and inflam-
mation. Of particular interest, the increased phagocytic
response of keratinocytes correlates with increased activ-
ity of soluble serine proteases. Serine protease inhibitors
downregulated both constitutive and PAR2 stimulated
phagocytosis, suggesting that these proteases also acti-
vate PAR2 and thereby amplify the response. PAR2 is
upregulated in keratinocytes after ultraviolet exposure,
with implications for pigmentation (257).

4. Proliferation and wound healing

Several findings suggest that PAR1 could play a role
in wound healing. Activation of PAR1 stimulates prolif-
eration of keratinocytes (5, 251) and fibroblasts (45), and
also induce angiogenesis (285). Plasmin, thrombin, and
PAR1 AP induce expression of cysteine-rich angiogenic
protein Cyr-61, a growth factor-like gene implicated in
angiogenesis and wound healing, in fibroblasts from wild-
type but not PAR1-deficient mice (227). Moreover, the
topical application of thrombin and PAR1 AP to incisional
wounds in rats improves wound strength and increases
angiogenesis, thereby promoting wound healing (36). The
role of PAR1 in wound healing has been examined in
PAR1-deficient mice (65). Although fibroblasts from these

E. Protease Signaling in the Skin

The role of proteases in cutaneous biology and dis-
ease has recently been reviewed (234).

1. Proteases

Cells in the epidermis and dermis could be regulated
by proteases of the coagulation cascade. Inflammation of
the skin is associated with an influx of inflammatory cells
that release proteases that activate PAR2. Thus whereas
in normal skin mast cells are found in the dermis, in
atopic dermatitis and psoriasis there is an influx of
tryptase-containing mast cells in the dermal and epider-
mal junction, as well as the epidermis (268). In addition,
epidermal cells express proteases, some of which could
activate PARs. Potential activating enzymes include stra-
tum corneum trypsic enzyme and chymotryptic enzyme
(96, 275). However, it remains to be determined if pro-
tases such as these activate PARs. Exogenous proteases
from mites, bacteria, and fungi could also signal to epi-
dermal cells.

2. PARs

Immunoreactive PAR2 is found in the basal and spi-
nous layers of the human epidermis but not in the gran-
ular layer, and human basal keratinocytes and a keratin-
ocyes cell line express PAR2 mRNA and protein (76).
PAR2 immunoreactivity is predominantly localized to the
basal and suprabasal spinous layers, although the stratum
cornueum is unstained (76, 268). PAR2 is also expressed in
the inner root sheath of hair follicles and in myoepithelial
cells of sweat glands. Keratinocytes in culture also ex-
press PAR2 mRNA and protein (251). PAR2 mRNA is also
moderately expressed in cultured human microvascular-
ture endothelial cells, but melanocytes do not express

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animals do not respond to thrombin, the time to closure of open skin wounds and the incision strength of healed wounds is unaffected by deletion of PAR$_1$. The discrepancy between this observation and the finding that PAR$_1$ AP promotes healing in rats is difficult to explain, but could be related to differences in species and the wound model chosen.

5. Inflammation

Activation of PAR$_1$ and PAR$_2$ induces expression of interleukin-6 and -8 as well as GM-CSF in cultured human keratinocytes, indicating that serine proteases modulate cytokine expression in the epidermis (126, 307). GM-CSF mediates Langerhans cell maturation, activates tissue macrophages, and stimulates proliferation of keratinocytes. PAR$_2$ agonists also activate stress-activated protein kinases (c-Jun amino-terminal kinase or JNK and p38) and the NFkB pathways in the keratinocyte cell line NCTC2544 (145). The NFkB family of transcription factors is implicated in regulation of a number of proinflammatory genes in these cells.

The proinflammatory effects of PAR$_2$ have also been examined in the intact animal. PAR$_2$-deficient mice exhibit diminished ear swelling and infiltration of inflammatory cells in a model of allergic dermatitis, suggesting that PAR$_2$ mediates allergic dermatitis (159). PAR$_2$ activators also signal to primary spinal afferent neurons that innervate the skin, resulting in the release of the neuropeptides SP and CGRP, which together induce neurogenic inflammation (216) (Fig. 8). This signaling also triggers a prolonged mechanical and thermal somatic hyperalgesia, which is due to the release of SP from central projections of these neurons within the dorsal horn of the spinal cord (296). Protease signaling to sensory nerves in the skin has recently been investigated in humans (234). Atopic dermatitis is associated with an elevated release of tryptase in the skin and an upregulation of PAR$_2$ on nerve cells. Cutaneous administration of PAR$_2$ AP to these patients results in prolonged itch, even in the presence of antihistamines drugs (269). These results suggest that tryptase could act on PAR$_2$-expressing sensory nerves in human skin to produce itch, which may explain the insensitivity of these patients to antihistamines.

VI. PROTEASE-ACTIVATED RECEPTORS: MECHANISMS OF DISEASE AND TARGETS FOR THERAPY

Proteases and PARs make important contributions to disease and are targets for therapies. First, PARs regulate many biological processes that are critical in disease, including coagulation, proliferation and survival, inflammation, neurotransmission, and pain. Second, proteases that activate PARs are generated during diseases, for example, in trauma, hemostasis, inflammation, and tumor formation. Third, the expression and function of PARs are altered in disease. Thus PAR$_2$ is upregulated during inflammation (216), and a polymorphic variant of PAR$_2$ has been identified with markedly abnormal sensitivity to PAR agonists (61). Finally, studies of genetically modified animals and using PAR antagonists and protease inhibitors suggest a role of PARs in models of diseases.

However, there are formidable obstacles toward understanding of the role of proteases and PARs in disease. A major problem is the lack of widely available antagonists; although PAR-deficient mice are available, mice are not always the optimal species to model human diseases. Another problem is the apparent redundancy of the PAR system. Cells express several PARs for the same protease, and several proteases can activate the same receptor. Effective blockade of protease signaling may require antagonism of several PARs or proteases. Finally, the roles of PARs have been specifically studied in few animal models of disease. Moreover, little is known about the expression of proteases and PARs in diseased human tissues. With these caveats in mind, the role of PARs will be briefly discussed in several diseases.

A. Cardiovascular Disease

The inappropriate aggregation of platelets makes an important contribution to occlusive vascular disorders such as stroke, angina, and myocardial infarction, where accumulation of atherosclerotic plaques promotes platelet-mediated thrombus formation. Thrombin is a major mediator of platelet aggregation and fibrin deposition, and thrombin inhibitors are useful antithrombotic agents. One drawback of such inhibitors is their disruption of the normal hemostatic mechanism, and a selective suppression of the effects of thrombin on platelets may be advantageous. A difficulty of studying the role of PARs in thrombus formation is that platelets express several thrombin receptors, which vary among species. However, despite the redundancy and interspecies differences, recent observations in cynomologus monkeys, whose platelets, like those of humans, express PAR$_1$ and PAR$_4$, suggest that PAR$_1$ antagonists may be useful therapeutic agents in humans (88). Thus the PAR$_1$ antagonist RWJ-58259 markedly inhibits thrombus formation and vessel occlusion in a model of electrolytic injury of the carotid artery in cynomologus monkeys. Despite the fact that platelets from these animals possess the dual PAR$_1$/PAR$_4$ system, antagonism of a single receptor is effective, raising the possibility that antagonism of a PAR$_1$ may be of use for treatment of thrombosis disorders in humans. In guinea pigs, whose platelets express three thrombin receptors (PAR$_1$, PAR$_3$, and PAR$_4$), RWJ-58259 is only marginally effective in arterial thrombosis, which emphasizes the
importance of selecting the appropriate species to model human disease (8).

PAR antagonists may also be of interest in treating vascular injury. In addition to its role in thrombus formation, thrombin is also implicated in restenosis of the blood vessels after vascular injury. Thus a PAR1 blocking antibody or a PAR1 antagonist attenuates thickening of the neointima of the rat carotid artery rat, initiated by damage to the endothelium by balloon angioplasty (8). Similarly, vascular injury is attenuated in PAR1-deficient mice (301). The protective effect of PAR1 antagonism or deletion occurs even though rat and mouse platelets do not express PAR1 and thus exhibit normal aggregation in the absence of functional PAR1. These effects of thrombin on restenosis are therefore dependent on the proliferative or proinflammatory effects of PAR1 on vascular smooth muscle.

PAR2 may contribute to injury that follows ischemia and reperfusion of tissues (203). Ischemia and reperfusion of the heart induces injury that is characterized by generalized inflammation and necrosis. PAR2 is upregulated in a model of coronary ischemia and reperfusion injury in rats, and administration of a PAR2 AP markedly protects the heart from injury. PAR2 activation also improves efficiency of ischemic preconditioning and reduces cardiac inflammation in the rat heart (204).

B. Inflammatory Disease

There is considerable interest in understanding the role of PARs, in particular PAR2, in inflammatory diseases, and this topic has been thoroughly reviewed (54, 57, 69). Observations on PAR2-deficient mice suggest a role for this receptor in inflammation of the airway, joints, and kidney. In allergic inflammation of the airway induced by immunization and challenge with ovalbumin, there is diminished infiltration of eosinophils and reduced hyperreactivity to methacholine in PAR2-deficient animals, whereas these responses are exacerbated in mice overexpressing PAR2 (255). Rheumatoid arthritis is a chronic inflammatory condition that is associated with inflammation of the synovium and hyperplasia. There is also elevated expression of TF and thrombin in the synovium and the deposition of fibrin in the inflamed joint, indicating activation of the coagulation system. Immunization and subsequent challenge of mice with chicken collagen results in arthritis, and administration of the thrombin inhibitor hirudin reduces the severity of the inflammation, assessed by clinical scoring and measurement of expression of proinflammatory cytokines, providing direct evidence for the involvement of thrombin in the inflammation (186). Evidence for a role of PAR2 in arthritis has been provided from observations of PAR2-deficient animals, which are protected against arthritis induced by intra-articular and periarticular injection of adjuvant (101). This inflammation is accompanied by an upregulation of PAR2 expression, which is normally confined to the vasculature of the joints. The protease that activates PAR2 in the inflamed joint remains to be determined, although it is tempting to speculate that FVIIa and FXa could be involved in light of the established activation of the coagulation cascade in arthritis. Proteases and PARs may also contribute to renal inflammation. Crescents glomerulonephritis is a severe and progressive form of renal inflammation that is a common cause of end-stage kidney disease. It is associated with fibrin deposition, suggesting activation of the coagulation proteases. Immunization with sheep globulin followed by challenge with sheep-anti-mouse glomerular basement membrane induces glomerulonephritis in mice that is characterized by fibrin deposition, infiltration of leukocytes, and reduced glomerular filtration (75). This inflammation is markedly diminished by treatment with the thrombin inhibitor hirudin, and inflammation is reduced in PAR1-deficient mice, indicating involvement of coagulation proteases and PAR1.

The observation that inflammation is diminished in PAR-deficient animals suggests that PAR antagonists and protease inhibitors may be useful anti-inflammatory agents. Indeed, trypsin inhibitors have been used to treat asthma and inflammatory bowel disease in humans (170, 284). However, it must be emphasized that PARs can exert anti-inflammatory and protective effects in certain conditions. Thus administration of recombinant APC blocks apoptosis in human brain endothelial cells and is neuroprotective in an animal model of ischemic brain injury (46). Moreover, APC reduces mortality of patients with sepsis (20). These protective actions of APC on endothelial cells are dependent on PAR1 (46, 238). In addition, activation of PAR2 induces bronchodilatation (56), inhibits lipopolysaccharide-induced pulmonary neutrophilia (195), diminishes formation of gastric ulcers (153) and experimental colitis (103), and protects against ischemia-perfusion injury in the heart (203). Thus agonists of PAR1 and PAR2 could be useful therapies for certain conditions.

C. Cancer

The microenvironment of tumors is replete with proteases that can activate PARs, and tumor cells themselves express PARs. Malignant cells secrete thrombin and trypsin, which can affect proliferation and mediate metastatic processes such as cellular invasion, extracellular matrix degradation, angiogenesis, and tissue remodeling.

PAR1 and PAR2 are expressed by a wide range of tumor cells (24, 80, 82, 208, 316). In breast tumor tissues, PAR1 and PAR2 are expressed in the tumor cells, mast cells, macrophages, endothelial cells, and vascular...
smooth muscle cells of the metastatic tumor microenvironment (80). In particular, there is an upregulation of PAR1 and PAR2 in proliferating stromal fibroblasts surrounding the carcinoma cells. In pulmonary tumor alveolar walls, the expression of PAR1 and PAR2 mRNA is increased by 10- and 16-fold, respectively, compared with normal alveolar tissues (140). Expression of trypsin is also detected in this tumor tissue.

The metastasis of tumor cells requires cell detachment from the expanding tumor mass, degradation of the stromal tissue and basement membrane, and increased cell motility. There is considerable evidence that proteases and PARs may contribute to these processes. The level of expression of PAR1 on tumor cells directly correlates with metastatic potential in both primary breast carcinoma and established cancer cell lines (97). Introduction of PAR1 antisense cDNA inhibits the invasion of metastatic breast carcinoma cells in culture through a reconstituted basement membrane, indicating an important role for PAR1 in tumor cell migration. In support of this observation, thrombin and PAR1 AP promote the invasion of a highly aggressive breast cancer cell line expressing PAR1 in an in vitro assay (122). Exposure of certain tumor cells to thrombin and PAR1 AP also enhances their adhesion to platelets, fibronectin, and von Willebrand factor in vitro and promotes pulmonary metastasis when cells are administered to mice in vivo (209, 210). Moreover, overexpression of PAR1 in certain tumor cells can enhance their metastatic potential in animal models. In colon cancer cell lines, activation of PAR1 induces a marked mitogenic response, which is dependent on activation of MAP kinase ERK1/2, and also stimulates motility of wounded cells (81). Together, these results suggest that antagonists of PAR1 may be useful treatments for proliferation and metastases of certain tumors. However, PAR1 AP has also reported to inhibit migration and invasion of breast cancer cell lines when applied as a concentration gradient in the direction of movement (144). PAR2 may also contribute to tumor formation and metastasis since PAR2 AP stimulates proliferation of colon tumor cell lines (82).

VII. CONCLUSIONS AND FUTURE PERSPECTIVES

There have been substantive advances in our understanding of protease signaling. Four PARs have been cloned and characterized, and the mechanisms by which proteases cleave and activate PARs are understood in considerable detail. A number of mammalian and nonmammalian proteases have been identified that can activate PARs, providing new insights into protease signaling. The realization that anchoring proteins, including PARs themselves or other nonreceptor proteins, play a critical role as cofactors for protease signaling has led to the identification of new activating enzymes. PARs have been used as model receptors to investigate mechanisms of desensitization and downregulation of GPCRs in general. Tools have been developed to examine receptor functions, including specific agonists and antagonists and both knockout and transgenic mice. With the use of these tools, the contribution of PARs to disease has been investigated in experimental animals. Finally, protease inhibitors, for example, tryptase inhibitors, and proteases themselves, such as APC, have been used to successfully treat human diseases. The effectiveness of these therapies may well be attributable to effects on signaling by PARs, raising the possibility that antagonists and agonists of PARs may be useful drugs.

Despite this impressive progress, there is much to learn about proteases and their receptors. First, the proteases that activate PARs are not fully characterized. Given the extremely widespread distribution of PARs, it is almost certain that new activating enzymes await discovery. This omission is particularly true for PAR2; although trypsin is a very potent agonist of this receptor, almost nothing is known about the function and regulation of extrapancreatic trypsins, which are likely to activate PAR2 in certain tissues. Second, there is good evidence for the existence of other PARs. Many proteases have biological actions that appear to be receptor mediated but which cannot be explained by the known PARs. Moreover, differences in the rank order of potencies of PAR agonists in bioassays point to the existence of receptor subtypes. Whether these novel receptors are PAR-like GPCRs or different types of receptor remains to be determined. Third, the mechanisms of PAR signaling are not fully explored. Thus the process by which proteolytic cleavage of an extracellular portion of the receptor induces the interaction of intracellular domains with heterotrimeric G proteins is not understood. Furthermore, the molecular events that terminate this irreversible mechanism of activation are not completely defined. For example, little is known about the postendocytic sorting mechanisms that target PARs for degradation. Fourth, and most important, from the standpoint of physiology and medicine, the optimal tools to study the function of PARs, receptor antagonists, are generally lacking. Although antagonists have been reported for certain PARs, they are not widely available. Finally, information about the role of proteases and PARs in many experimental systems is simply lacking. It will be of great interest to investigate the contributions of proteases, protease inhibitors, cofactors, and PARs in experimental models of human disease using genetically modified animals and available pharmacological reagents.

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