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International Union of Pharmacology. XXVIII. Proteinase-Activated Receptors

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***Abstract*——Proteinase-activated receptors (PARs) represent a unique subclass of G-protein-coupled receptors of which four family members have now been cloned from a number of species. The novel mechanism of receptor activation involves the proteolytic unmasking of a cryptic N-terminal receptor sequence that, remaining tethered, binds to and triggers receptor function. In addition, short (five to six amino acids) synthetic peptides, based on the proteolytically revealed motif, can activate PARs without the unmasking of the tethered ligand. This article summarizes the experiments leading to the pharmacological characterization and cloning of the four PAR family members and provides a rationale for their designation by the acronym "PAR". The ability to distinguish among the PARs pharmacologically 1) with selective proteinase activators, 2) with receptor-selective peptide agonists, and 3) with peptide and nonpeptide antagonists is discussed, as are the molecular mechanisms of receptor activation and desensitization/internalization. Finally, the potential physiological roles of the PARs, which are widely distributed in many organs in the settings of tissue injury, repair, and remodeling, including embryogenesis and oncogenesis are discussed, and the newly appreciated roles of proteinases as signaling molecules that can act as either functional agonists or antagonists are highlighted.**

I. Introduction

Few receptor searches have revealed more intriguing receptor mechanisms than the one leading to the discovery of the receptors responsible for thrombin-mediated platelet aggregation. The search for a functional thrombin receptor, using expression cloning methods rather than the previously unsuccessful classical ligand-binding approach, led to the cloning of a G-protein-coupled receptor (Rasmussen et al., 1991; Vu et al., 1991a) that mediates the cellular actions of thrombin on platelets, endothelial cells, fibroblasts, and other cell types. The unique property of the

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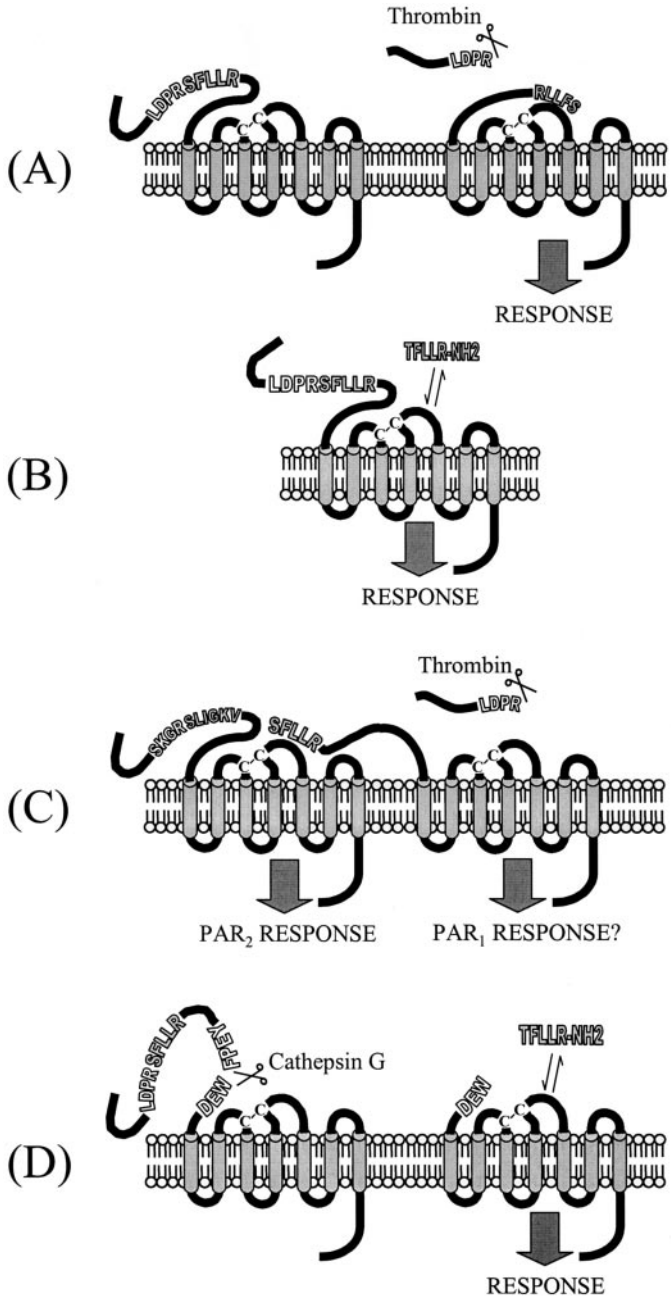

FIG 1. Mechanisms of activation and inactivation of PARs. A, the functional PARs cloned to date contain within their receptor N terminus a serine protease cleavage/activation site that, once cleaved by a serine protease, results in the unmasking of a cryptic tethered ligand sequence. The revealed tethered ligand sequence is believed to bind to and subsequently activate the receptor. The example provided (A) is for human PAR_1 activation by thrombin. B, PARs may be activated independently of proteolytic unmasking of the tethered ligand sequence by small synthetic peptides (PAR-APs) corresponding to the first five or six amino acids of the tethered ligand sequence. Activation by the receptor-selective PAR_1AP, TFLLR-NH_2, is shown. C, intermolecular activation of PAR_1 or PAR_2 may occur following PAR_1 activation, whereby the unmasked tethered ligand of a PAR_1 receptor activates an uncleaved neighboring PAR_2 or PAR_1 receptor. D, inactivation of PARs may occur by proteases cleaving the receptor C-terminally of the tethered ligand sequence, thus amputating the tethered ligand from the receptor body. These amputated receptors can no longer be activated by proteinases but are still responsive to exogenously applied PAR-APs. Note: a putative cysteine palmitoylation site in the C-terminal tail (present in all PARs except human PAR_4) may result in a fourth intracellular loop (not shown) that could play a role in receptor signaling.

thrombin receptor that distinguishes it from all other G-protein-coupled receptors relates to its lack of a "classical" circulating agonist and its unusual mechanism of triggering that involves the proteolytic unmasking of an N-terminal sequence buried in the "pro-receptor" (Fig. 1A), which then acts as a "tethered" or anchored receptor-stimulating ligand. Thus, in thinking about the proteinase-activated receptor (PAR^2) family, for which the first discovered receptor for thrombin is the prototype (now designated PAR_1), it is necessary to make a paradigm shift from the perspective of all other previously known G-protein-coupled receptor systems. Remarkably, it was demonstrated (Vu et al., 1991a) that without the unmasking of its cryptic tethered ligand, PAR_1 can be activated by relatively short synthetic peptides with sequences based on that of the proteolytically revealed tethered ligand (so-called thrombin receptor-activating polypeptides, or TRAPs; now referred to as PAR-activating peptides, or PAR-APs). The TRAPs (or PAR_1APs) were used as surrogate activators of PAR_1 to evaluate the potential actions of thrombin on tissues wherein the effects of the proteinase itself might be difficult to interpret; the PAR_1APs have also served as a basis for the development of PAR_1-targeted antagonists (see below).

Results with the originally designed PAR_1APs, including peptide structure-activity studies, interspecies studies of platelet aggregation, and the development of PAR_1 knockout mice, clearly pointed to the existence of other members of the PAR family. Thus, the serendipitous discovery of a second G-protein-coupled proteinase-activated receptor that could be stimulated by trypsin, but not thrombin (PAR_2; Nystedt et al., 1994), was not entirely unexpected. PAR_2 was found not in the course of a search for additional "thrombin receptors" but as a result of screening a mouse genomic library with a bovine substance K receptor oligonucleotide probe. The discovery of PAR_2 was facilitated by the concurrent presence in the protein/nucleotide database of the sequence of PAR_1. In addition to having considerable homology with PAR_1, PAR_2 exhibited the hallmark of PAR_1 in terms of the tethered ligand mechanism for its activation by trypsin. Like PAR_1, PAR_2 can also be activated by short peptides based on the N-terminal "tethered ligand" revealed by trypsin (e.g., SLIGKV or SLIGRL . . . from the sequences of human and rat PAR_2, respectively). Although the PAR_2-AP SLIGRL-NH_2 cannot activate PAR_1, we soon realized that the PAR_1APs originally described could activate PAR_2, and peptide structure-activity studies were required to design PAR_1APs that could selectively activate PAR_1 without activating

[2] Abbreviations: PAR, proteinase-activated receptor; PAR-AP, PAR-activating peptide; Cha, cyclohexylalanine; TRAP, thrombin receptor-activating peptide; NC-IUPHAR, International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification; NCBI, National Center for Biotechnology Information; NC-IUBMB, Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.

TABLE 1
Proteinase-activated receptors

IUPHAR recommended name	PAR_1	PAR_2	PAR_3	PAR_4
IUPHAR receptor code	2.1:PAR:1:PAR1:HUMAN:00	2.1:PAR:2:PAR2:HUMAN:00	2.1:PAR:3:PAR:3:HUMAN:00	2.1:PAR:4:PAR:4:HUMAN:00
Alternate designators	Thrombin receptor	Trypsin receptor	Thrombin receptor	Thrombin receptor
Receptor variants		F240S mutation (h)		
Amino acid composition	425 aa (h)[a]	397 aa (h)	374 aa (h)	385 aa (h)
Agonist proteinases	Thrombin > trypsin	Trypsin, tryptase, trypsin 2, matriptase/	Thrombin ≫ trypsin > factor	Thrombin ≅ Trypsin
Xa	Thrombin ≅ trypsin	MT-serine protease 1	Proteases cleavage but receptor does not generate a	Cathepsin G; factors
does	Cathepsin G; factors VIIa/X	Factor X_a/TF/VIIa, Der p3, Der p9	calcium signal	VIIa/X
Inactivating proteinases	Cathepsin G, proteinase 3, elastase, plasmin, chymase	Unknown	Unknown	Unknown
Tethered ligand sequences	SFLLR (h), SFFLR (m,r)	SLIGKV (h), SLIGRL (m,r)	TFRGAP (h), SFNGGP (m)	GYPGQV (h), GYPGKF (m)
Subtype selective peptide agonists	TFRIFD[b] TFLLR-NH_2	SLIGKV-NH_2, SLIGRL-NH_2 *Trans*-cinnamoyl-LIGRLO-NH_2		GYPGKF-NH_2[c] GYPGQV-NH^2 AYPGKF-NH_2
Antagonists	*Trans*-cinnamoyl-parafluoro-Phe-paraguanidino-Phe-Leu-Arg-Arg-NH_2[d]			
	Mercaptopropionyl-Phe-Cha-Arg-Lys-Pro-Lys-Pro-Asn-Asp-Lys-NH_2[f]			TRANS-cinnamoyl-YPGKF-NH_2[e]
	Non-peptide antagonists: RWJ56110 and RWJ58259			
Signal transduction mechanisms	$G_{q/11}$ (increased IP_3/DAG); G_i; $G_{12/13}$	—[g]	None known	—[g]
Gene/chromosome	F2R/5q13 (h)	F2RL1/5q13 (h)	F2RL2/5q13 (h)	F2RL3/19p12 (h)
	F2R/13D2 (m)	F2RL1/13D2 (m)	F2RL2/13D2 (m)	F2RL3/8B3.3 (m)
NCBI accession no.	M62424 (h)	U34038 (h)	U92971 (h)	AF080214, AF055917 (h)
	L03529 (m)	Z48043 (m)	U92972 (m)	AF080215 (m)
	M81642 (r)	U61373 (r)		

[a] The IUPHAR receptor code is provided for each human (h) PAR, but not for murine (m), or rat (r) PARs.
[b] TFRIFD is the *Xenopus* thrombin receptor tethered ligand domain (the human PAR_1 tethered ligand domain sequence, SFLLRN, activates both PAR_1 and PAR_2 receptors).
[c] AYPGKF is 10 times more potent than the natural human (GYPGQV) or murine (GYPGKF) tethered ligand sequences, which are also PAR_4 selective.
[d] May also act at PAR_2 as agonist; also designated BMS 200261.
[e] Validated only in rat platelets.
[f] Antagonizes PAR_1; acts as an agonist at PAR_2.
[g] Stimulates phosphositide turnover through an uncharacterized G protein, probably $G_{q/11}$; G_i interactions with PAR_2 not evaluated; PAR_4 believed *not* to interact with G_i.

PAR_2. The availability of peptide agonists selective for PAR_1 and PAR_2 (Table 1) clarified the distinct and overlapping actions that these two receptor systems can have in a variety of tissues (see below).

However, the discovery of PAR_1 did not explain either the lack of activity of PAR_1APs in affecting rat or rabbit platelets (Kinlough-Rathbone et al., 1993) or the activity of thrombin in murine platelets that lack PAR_1 (Connolly et al., 1996). The absence of PAR_1 in murine platelets prompted a continued search for more thrombin receptors, resulting in the discovery of the third family member, PAR_3 (Ishihara et al., 1997). Yet again, when thrombin was still found to affect platelets from PAR_3 −/− mice, a BLAST search of the NCBI expressed sequence tag database using the coding region of human PAR_2 was initiated, ultimately leading to the discovery of murine PAR_4 (Kahn et al., 1998). Human PAR_4 was discovered simultaneously, also as a result of a search of public and commercially available expressed sequence tag databases using human PAR 1, 2, and 3 sequences as "query" sequences (Xu et al., 1998). All of the cryptic tethered ligand sequences of the different PAR family members have turned out to be unique (Table 1). Unexpectedly, murine PAR_3 does not appear to signal itself via its thrombin-revealed sequence but, rather, acts as a cofactor for the activation of PAR_4 (see below and Nakanishi-Matsui et al., 2000). The physiological role for PAR_3 is at present somewhat of an enigma. Synthetic peptides modeled on the thrombin-revealed sequence of PAR_3 (e.g., TFRGAP. . .) do not activate murine PAR_3 but may activate both PAR_1 and PAR_2. The PAR_4 tethered ligands (murine, GYPGKF and human, GYPGQV) fail to activate other PARs but are of limited value as agonists for physiological studies because of their low potencies (active in the 100–400 μM range). For activation of PAR_4, the more potent peptide analog, AYPGKF-NH_2, appears more promising than the parent tethered ligand sequences (Faruqi et al., 2000). Table 1 shows a number of potentially useful selective peptide agonists for the several PARs.

In summary, to date, four proteinase-activated receptors that are regulated by a proteolytically revealed tethered ligand mechanism (Fig. 1) are now known (Table 1). The PARs belong to family 1 of the G-protein-coupled superfamily of receptors, branching off from a relatedness to the neurokinin, substance P, luteinizing hormone- and thyroid-stimulating hormone receptors (Vu et al., 1991a). It is the object of the sections that follow, to provide a current synopsis of the molecular pharmacology and physiology of these recently discovered receptors along with a rationale for the recommended nomenclature. Because of the novel features of this relatively new receptor family, more information will be included in this article than may be found in other IUPHAR receptor nomenclature summaries. Although many examples will be provided, the coverage is meant to be an overview rather than an all-inclusive treatise. Thus, at the outset, we offer our apologies for not citing quite a number of important contributions that were omitted in an attempt to condense the text. For more comprehensive information, the reader is referred to a number of current synopses and more exhaustive review articles (Dery et al., 1998; Coughlin, 2000; Macfarlane et al., 2001; O'Brien et al., 2001; Vergnolle et al., 2001b).

II. Nomenclature and Use of the Proteinase-Activated Receptor Designation

As pointed out under *Section I.*, the hallmark that singles out the PARs from other G-protein-coupled receptor systems is the unique proteolytic activation mechanism that reveals a cryptic amino-terminal tethered ligand. Although initially PAR_1 was termed the "thrombin receptor", it was known at the time of PAR_1 discovery (Rasmussen et al., 1991; Vu et al., 1991a) that thrombin could trigger some cell responses via a process distinct from its proteolytic activation mechanism, involving at least two different mitogenic/chemotactic peptides (thrombin-derived peptides) originating from the sequence of thrombin itself (Bar-Shavit et al., 1986; Glenn et al., 1988). This issue was initially confused further by the use of the acronym TRAP for both those peptides activating what is now termed PAR_1 (so-called PAR_1APs) and peptides that interact with the as yet unidentified receptor(s) for the thrombin-derived peptides. To render the issue of nomenclature more complex, it can be pointed out that other receptor systems, like the one for insulin, can be activated by a proteolytic mechanism that does *not* involve the unmasking of a cryptic receptor-activating ligand (reviewed briefly by Hollenberg, 1996). Thus, the term PAR, adopted by the NC-IUPHAR committee (Table 2), refers specifically to the G-protein-

TABLE 2
IUPHAR Nomenclature Committee on PARs

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Johan Sundelin	Division of Molecular Neurobiology, Lund University, Lund, Sweden
Ellen Van Obberghen-Schilling	Institute of Signaling, Developmental Biology and Cancer, CNRS UMR6543, Centre A. Lacassagne, Nice, France

coupled receptor subfamily that is activated proteolytically via a tethered ligand mechanism. The designation PAR was retained because, historically, the acronym has been widely used in the literature in a variety of fields (hematology, vascular biology, developmental biology, and neurobiology) dealing with the biological actions of thrombin and trypsin. It can be noted that, since a variety of proteinases can regulate PARs (see below and Table 1), the selected PAR abbreviation has the virtue of conveying generalized mechanistic and functional information. Furthermore, the "R" in PAR has been retained for receptor designation to avoid confusion with the acronym "PA", used frequently for the enzyme plasminogen activator. Notwithstanding, the unwary who regularly search the database with this designated "PAR" acronym will find that the abbreviation has already been claimed in a number of other fields, ranging from *Caenorhabditis elegans* genetics to prostate cancer biology. Finally, there is the unforgiving computer algorithm, which will yield a distinct database depending on whether one uses the biochemically correct definition, "proteinase", or the commonly used term "protease". Because PARs are activated by an endoproteolytic cleavage, the term "proteinase," commonly used to designate the NC-International Union of Biochemistry and Molecular Biology Nomenclature Committee-recommended term, "endopeptidase" (discussed by Barrett, 2001), is more precise to describe these receptors. In summary, the designated receptor nomenclature outlined in Table 1 for the PAR family is appropriate and in common usage for describing those receptors that possess a proteolytically activated tethered ligand mechanism, but the acronym "PAR" must be used with care for an efficient general literature search.

III. Receptor Subtypes

A. Defining Proteinase-Activated Receptor Subtypes Using Enzyme and Peptide Agonists

A traditional pharmacological approach to receptor classification (e.g., Ahlquist, 1948) did indeed point to the existence of PAR subtypes (Hollenberg et al., 1993) well before the cloning of all four currently known family members listed in Table 1. As mentioned above, the use of PAR_1APs, and thrombin itself, as receptor probes pointed to a unique set of PARs present on rodent platelets before the successful cloning of PAR_3 and PAR_4. Furthermore, upon discovery of PAR_2, it became clear that although trypsin at appropriate concentrations could activate both PAR_2 (<10 U/ml; approximately 20 nM) and PAR_1 (≥50 U/ml; >100 nM), thrombin could not activate PAR_2. Thus, pharmacologically, the PARs can be distinguished on the basis of their relative susceptibility to activation by serine proteinases (principally, thrombin and trypsin), and there is every reason to expect that the use of distinct proteinase families as probes may lead to the discovery of novel proteinase-activated receptor systems that may or may not use a tethered ligand mechanism for activation. Apart from proteinase susceptibility, the most clear-cut molecular characteristic that distinguishes one PAR from another is the sequence of the proteolytically revealed tethered ligand (Table 1). As alluded to above, studies with peptide analogs of the PAR_1APs pointed clearly to the presence of a distinct receptor system in rat gastric and vascular tissues that turned out to be PAR_2 (Hollenberg et al., 1993; Al-Ani et al., 1995). Thus, the PAR-APs have proved to be particularly useful receptor agonist probes. It has been possible to develop peptide agonists that readily distinguish among the four receptor subtypes (Table 1) and can be used as agonists to assess the potential physiological roles that each of the four receptor systems may play in vivo (see below). That said, although such peptides can be shown to be receptor-selective in cell and tissue expression systems (e.g., see Blackhart et al., 1996; Hollenberg et al., 1999; Kawabata et al., 1999), the comparatively low potency of the PAR-APs (active in the 1–400 μM range), especially for some of the low-potency PAR_4APs (active in the 100–400 μM range), should sound a cautionary note. It is entirely feasible that such PAR-APs could, at elevated concentrations in vivo, activate receptors other than the PARs (e.g., see Roy et al., 1998). Notwithstanding, the use of selected proteinases and selective PAR-APs can serve to distinguish among the PAR subtypes cloned to date.

B. Receptor Antagonists and Receptor Subtypes

The development of receptor-selective antagonists for the PAR family has proved to be an enormous challenge. Initially, the search for antagonists was based on structure-activity studies using the sequence of human PAR_1 (SFLLRN) as a point of departure (Scarborough et al., 1992; Vassallo et al., 1992; Rasmussen et al., 1993; Ceruso et al., 1999), without any knowledge of the existence of receptor subtypes. One of the first such antagonists to be reported (Seiler et al., 1995) based on the SFLLRN motif, 3-mercaptopropionyl-Phe-Cha-Cha-Arg-Lys Pro-Asn-Asp-Lys-amide, was found to block thrombin action on human platelets at low (e.g., <0.05 U/ml) but not high concentrations, possibly because of the then unknown presence of PAR_4 on human platelets. This same compound can act as a PAR_2 agonist. A second PAR_1 peptide antagonist, *trans*-cinnamoyl-parafluoro-Phe-paraguanidino-Phe-Leu-Arg-amide (BMS 200261, also based on the PAR_1 SFLLRN. . . sequence), has also been described (Bernatowicz et al., 1996), which will very likely also act as a PAR_2 agonist, since the peptide *trans*-cinnamoyl-Leu-Ile-Gly Arg-Leu-Orn-amide acts as a relatively potent activator of PAR_2 (Vergnolle et al., 1998). Interestingly, the peptide *trans*-cinnamoyl-Tyr-Pro-Gly Lys-Phe-amide, based on the murine PAR_4 tethered ligand sequence, can act as a thrombin antagonist in rat platelets (Hollenberg and Saifeddine, 2001; Ma et

al., 2001), but the ability of this peptide to block PAR_4 in other species remains to be verified.

Nonpeptide PAR_1 antagonists have also been developed based on an analysis of the molecular conformation of the PAR_1-activating peptide, SFLLRN (Ceruso et al., 1999). The screens for developing PAR_1 antagonists have used either functional assays (e.g., thrombin or PAR_1AP-mediated platelet aggregation or calcium signaling) (e.g., Andrade-Gordon et al., 1999; Barrow et al., 2001) or a ligand-binding assay (Ahn et al., 1997, 2000). Surprisingly, the functional inhibition of PAR_1 by an antagonist depends on whether a peptide or thrombin itself is used as an agonist (see below). The use of a peptide modeling approach along with a calcium signaling assay employing thrombin as an agonist led to the synthesis of the PAR_1 antagonist, RWJ56110, based on a 1,3,6-trisubstituted indole template (Andrade-Gordon et al., 1999). This compound behaves as a pure PAR_1 antagonist and blocks activation of the receptor by both thrombin and PAR_1APs. This work has led to the development of a second generation antagonist, RWJ58259, based on an indazole rather than an indole template, that has proved more favorable for use in vivo in a balloon angioplasty model of restenosis (Andrade-Gordon et al., 2001; Zhang et al., 2001). Preliminary information [summarized by Rotella (2001) and Chackalamannil (2001)] indicates that a number of other groups are also developing PAR_1 antagonists; one based on derivatives of 2-amino-4-(3,5-difluorophenyl) isoxazole, which blocks both peptide- and thrombin-induced receptor activation with IC_{50} values in the 0.1 μM range, and another based on the natural product, himbacine, which exhibits IC_{50} values in the 10-nM range in a ligand-binding assay. One looks forward to the complete characterization of these new PAR_1 antagonists. Thus, on the whole, it can be said that the development of selective nonpeptide antagonists for PAR_1 is still in its infancy, and it may be some time before such compounds are readily available for the routine evaluation of the role of PAR_1 in physiological processes. The development of effective antagonists for PAR_2 and PAR_4 is still just on the horizon (Hollenberg and Saifeddine, 2001). Notwithstanding, one can predict that PAR antagonists will play a key role in defining the physiological processes that the PARs subserve in vivo.

C. Molecular Definition of Receptor Subtypes

As outlined above (*Section III.A.*), the best pharmacological distinction among the four known receptor subtypes can be made using receptor-selective PAR-APs, based on the distinct sequences of the revealed tethered ligands. However, the definitive identification of the four PAR subtypes (Table 1) has come from the cloning and sequencing of the four distinct receptors and from studies of mice in which one or other of the receptors has been genetically eliminated. Northern blot analysis has revealed the presence of all four receptor subtypes in a wide range of tissues and cells in which their physiological roles are largely unknown (Table 3). PAR_3, as mentioned above, appears to be an unusual puzzle in that murine PAR_3 does not generate a signal on its own but appears to act as a cofactor for the activation of PAR_4 (Nakanishi-Matsui et al., 2000; Sambrano et al., 2001). Whether the distinct receptor subtypes cleaved by thrombin (PAR_1, PAR_3, and PAR_4) can play independent physiological roles remains to be determined. PAR_2, activated by trypsin, tryptase, and other serine proteinases, very likely plays a role complementary to the one(s) subserved by PAR_1, PAR_3, and PAR_4 (Nystedt et al., 1994; Böhm et al., 1996b; see below). Now that the receptor subtypes can be identified by molecular probe analysis (in situ hybridization, reverse transcription-polymerase chain reaction, and site-targeted antisera), their presence and actions in a variety of tissues can be explored using both a biochemical and pharmacological approach.

IV. Molecular Aspects of Proteinase-Activated Receptor Activation

A. The Tethered Ligand Mechanism

One of the first challenges upon discovery of the PAR_1 prototype of this receptor family (Rasmussen et al., 1991; Vu et al., 1991a) was to determine its mechanism

TABLE 3
Localization and potential roles of the human proteinase-activated receptors

	PAR_1	PAR_2	PAR_3	PAR_4
Tissue distribution[a] (Northern blot analysis)	Brain, lung, heart, stomach, colon, kidney, testis	Prostate, small intestine, colon, liver, kidney, pancreas, trachea	Heart, kidney, pancreas, thymus, small intestine, stomach, lymph node, trachea	Lung, pancreas, thyroid, testis, small intestine, placenta, skeletal muscle, lymph node, adrenal gland, prostate, uterus, colon
Cellular expression	Platelets, endothelium, vascular smooth muscle, leukocytes, GI tract epithelium, fibroblasts, neurons, mast cells	Endothelium, leukocytes, GI tract epithelium and lung, airway and vascular smooth muscle, neurons, mast cells, keratinocytes, lung fibroblasts, renal tubular cells	Airway smooth muscle, platelets	Platelets, megakaryocytes
Known physiological role	Platelet activation			Platelet activation
Potential physiological roles	Pro-inflammatory, embryonic development, regulation of vascular tone	Pro-inflammatory, mediator of nocioception, airway protection, regulation of vascular tone	Cofactor for PAR_4	Platelet activation

GI, gastrointestinal.
[a] Information for PAR_2, PAR_3, and PAR_4 is provided for human tissues; for PAR_1, distribution is recorded in rat tissues, for which more extensive data are available than in human tissues.

of activation. Noting the homology of the N-terminal PAR_1 sequence, $LDPR^{41}S$, with the LDPRI sequence responsible for thrombin-mediated activation of protein C, Vu et al. (1991a) established arginine-41 as the main target on PAR_1 for thrombin cleavage, revealing the sequence S^{42}FLLRNPNDKYEPF. . . upstream of a hirudin-like negatively charged sequence (WEDEEKNES) that serves to increase the binding affinity of thrombin for PAR_1 via the anion-binding exosite domain of thrombin (Vu et al., 1991b). This negatively charged sequence distal to the cleavage/activation site, to which the anion-binding exosite domain of thrombin can interact, is also present in PAR_3 but is absent from either PAR_4 or PAR_2. The presence of such a negatively charged sequence may rationalize in part the selectivity of thrombin for cleaving PAR_1 and PAR_3. Vu et al. (1991a) hypothesized that the revealed sequence, SFLLR. . . , might act as an activator of the receptor. Support of this tethered ligand hypothesis, involving an intramolecular mechanism for the activation of PAR_1 by thrombin, was provided not only by discovering the platelet-activating ability of the putative revealed sequence (SFLLRNPNDKYEPF) as a soluble peptide (Vu et al., 1991a) but also by experiments showing that substitution of the putative thrombin cleavage/activation site (LPDR/S. . .) with a cleavage target sequence for enterokinase (DDDDK/S), just upstream from the postulated tethered ligand receptor-activation sequence, resulted in a receptor that could be activated by enterokinase rather than thrombin (Vu et al., 1991b). Moreover, mutating the cleavage/activation site to make it resistant to thrombin proteolysis blocked thrombin-induced receptor activation. Furthermore, in confirming the intramolecular tethered ligand mechanism, it has been demonstrated that a proteolytically revealed ligand on PAR_1 can potentially activate a neighboring PAR (Chen et al., 1994; O'Brien et al., 2000) via an intermolecular mechanism, albeit with lesser efficiency. A thoughtful summary of these intermolecular activation mechanisms for the PARs is found in the review by O'Brien et al. (2001). The tethered ligand mechanism is presumed to activate PAR_2 and PAR_4 since the synthetic peptides based on the revealed sequences are able to activate the two receptors and since for PAR_2, rendering the activation sequence resistant to trypsin cleavage blocked receptor activation (Nystedt et al., 1994). As emphasized above, this tethered ligand mechanism has become the hallmark characteristic of the receptor family. The lack of signal generation by murine PAR_3 itself, which can also be cleaved to reveal a tethered ligand, remains to be explained. It can be suggested that the revealed tethered ligand on PAR_3 may activate other PARs via an intermolecular process.

B. Structure-Activity Relationships for Receptor Activation by the Tethered Ligand Sequences

As already mentioned (*Section III.A.*), considerable work has been done to explore the structure-activity relationships for the synthetic PAR-APs, based on the distinct tethered ligand sequences of PAR_1, PAR_2, and PAR_4 (Hollenberg et al., 1992, 1993; Scarborough et al., 1992; Vassallo et al., 1992; Natarajan et al., 1995; Faruqi et al., 2000; Maryanoff et al., 2001) (see also Table 1). Surprisingly, very short peptide sequences, only five or six amino acids in length, are sufficient to activate the PARs. Taken together, the structure-activity data obtained with PAR-APs for all of the PARs point to the key importance of the first two amino acid residues of the revealed tethered ligand sequence. For instance, an aromatic residue at position 2, e.g., phenylalanine, is required for the activity of PAR_1APs (e.g., see Natarajan et al., 1995), and a hydrophobic side chain (e.g., leucine or phenylalanine) appears to be required for the activation of PAR_2 by its tethered ligand sequence (Blackhart et al., 1996; Hollenberg et al., 1996; Maryanoff et al., 2001). Furthermore, acylating the N-terminal amino acid of a PAR-AP or merely reversing the order of the first two amino acids [e.g., FSLLR (inactive) versus SFLLR (active) for PAR_1; or LSIGRL (inactive) versus SLGRL (active) for PAR_2] abolishes the ability of the peptides to activate the receptor but does not lead to antagonist activity. Interestingly, substituting a *trans*-cinnamoyl group for the first amino acid (serine) of either the PAR_1- or PAR_4-activating peptides (but not for PAR_2APs) leads to peptides with antagonist activity for PAR_1 and PAR_4 (Bernatowicz et al., 1996; Hollenberg and Saifeddine, 2001; Ma et al., 2001). For the PARs, one can thus envision an interaction between complementary groups on the receptor with the positively charged N-terminal amino group, along with the hydrophobic side chain of the amino acid in the second position of the revealed tethered ligand sequence. Other residues, such as a positively charged lysine or arginine group, found at position 5 of all but one (human PAR_4) of the tethered ligand sequences for mammalian PARs 1, 2, and 4 have also been found to be important for the activity of PAR-APs. That said, the tethered ligands of *Xenopus* PAR_1 (TFRIFD. . .) and human PAR_4 (GYPGQV. . .) exhibit a key hydrophobic side chain at position 2 but do not possess a positively charged side chain at position 5. Yet, these peptides are capable of activating human PAR_1 and PAR_4 (albeit with very low potency) (Gerszten et al., 1994; Nanevicz et al., 1995; Faruqi et al., 2000). In this regard, it is interesting to note that a peptide, AFLARAA, which has the hydrophobic and basic side chains of a PAR_1AP as its main features, is a PAR_1 antagonist (Pakala et al., 2000). This peptide might also affect the other PARs.

To make matters more complex, it now appears that the structural requirements for the synthetic peptide sequences acting as free peptides to activate the receptor may differ somewhat from the structure-activity relationships for comparable peptide sequences acting as receptor-tethered ligands. As one example of this situation, Oksenberg and colleagues (Blackhart et al., 2000)

found that a mutation in an extracellular domain of human PAR_1 that abrogated the ability of the PAR_1AP, SFLLRNP-amide, to activate the receptor (without abolishing the ability of the receptor to bind the peptide) surprisingly did not affect the ability of thrombin to activate the mutated receptor by revealing the same peptide sequence (SFLLRNP. . .) as a tethered ligand. A complementary approach with PAR_2 has shown that a mutation introducing a positive charge into extracellular loop 2 of PAR_2 (see below) markedly reduced the potency of PAR_2 agonists acting as free peptides (e.g., SLIGRL-amide) but had little effect on the potency of trypsin to activate the receptor by unmasking the same sequence as a tethered ligand (Al-Ani et al., 1999). Thus, the conventional thinking about structure-activity relationships in the setting of G-protein-coupled receptors that respond to blood-borne agonists or neurotransmitters (e.g., muscarinic or adrenoreceptor systems) may have to be modified for the analysis of the tethered ligand proteolytically activated PAR family. The differences between the tethered ligand receptor docking mechanism and that of soluble agonists may have important implications for the design of nonpeptide PAR antagonists. In this respect, it is of interest that the PAR_1 antagonist RWJ56110 blocks receptor activation by both thrombin and PAR_1APs (Andrade-Gordon et al., 1999).

C. Receptor Domains Involved in Ligand Activation

Like all other pharmacological receptors, the PARs must perform the dual function of: 1) recognizing the activating ligand (in this case, recognition of both the activating proteinase and the revealed tethered ligand) and 2) triggering an intracellular response. Additionally, since PARs are activated by a tethered ligand that cannot diffuse away, this receptor family must also provide for a rapid shut-off/desensitization mechanism. Insight about the PAR domains involved in ligand activation have come from studies of PAR_1 chimeras (*Xenopus* PAR_1/human PAR_1 and human PAR_1/murine PAR_2) in which the sequence of extracellular loop 2 was found to confer peptide specificity for the activation of the receptor (Fig. 2). Thus, substitution of the human PAR_1 extracellular loop 2 (see Fig. 2) into *Xenopus* PAR_1 conferred on the chimeric receptor a sensitivity toward the human PAR_1AP, SFLLRN-amide, which was otherwise essentially inactive in the wild-type *Xenopus* PAR_1 (Gerszten et al., 1994; Nanevicz et al., 1995); and substitution of the extracellular loop 2 sequence of murine PAR_2 into human PAR_1 conferred on the chimeric receptor sensitivity to the PAR_2-AP, SLIGRL-amide, which does not activate human PAR_1 (Lerner et al., 1996). In addition, the data of Lerner et al. (1996) point to a possible interaction between the N-terminal extracellular domain of PAR_2 and extracellular loop 3 (see Fig. 2). Furthermore, site mutations in extracellular loop 2 have indicated the importance of the acidic residues, Glu-260 in human PAR_1 and Glu-232/Glu-233 of rat PAR_2, for receptor activation by PAR-APs (Nanevicz et al., 1995; Al-Ani et al., 1999). It has been suggested that there may be an electrostatic interaction between these acidic groups in extracellular loop 2 and the basic arginine side chain at position 5 of the synthetic PAR-APs, providing for a "gating" or direct interaction for receptor activation (Nanevicz et al., 1995; Al-Ani et al., 1999). Other acidic groups in the extracellular domain of human PAR_1, one in loop 2 (Asp-256) and another in the third extracellular loop (Glu-347), have also been found to play a role in receptor activation by synthetic PAR-APs (Blackhart et al., 2000). Furthermore, a short amino-terminal sequence (residues 83 to 93) in human PAR_1 (but not in PAR_2) has been found to play a role in receptor activation by peptide agonists (Bahou et al., 1994; Nanevicz et al., 1995; Lerner et al., 1996). An important role for other nonacidic residues in extracellular loop 2 of PAR_2

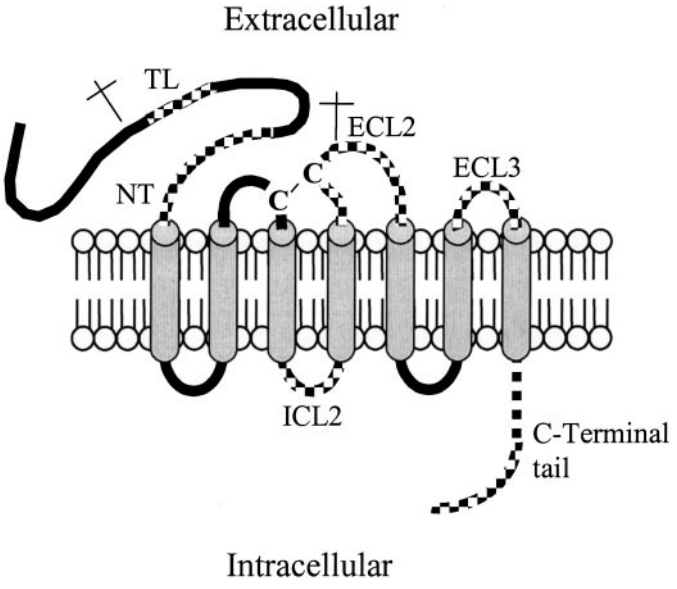

FIG 2. Schematic diagram of a PAR with domains thought to play a role in receptor signaling shown by checkering. The diagram is an amalgamation of the data obtained to date with PAR_1 and PAR_2. There is limited information on PAR_3 and PAR_4. For all of the PARs, with the exception of PAR_3, the tethered ligand (TL) is central to receptor activation following proteinase unmasking. Each tethered ligand exhibits specificity for its cognate receptor, except for PAR_1, where its TL peptide sequence also shows agonist activity toward PAR_2. For PAR_1 and PAR_2, extracellular loop 2 (ECL2) has been shown to confer PAR-AP specificity for the receptor, although the role of ECL2 for activation by the proteinase-revealed tethered ligand remains to be confirmed. For PAR_2, there is believed to be an important interaction between the N terminus (NT) and extracellular loop 3 (ECL3) in that substitution of the PAR_2 domains with comparable sequences for PAR_1 results in a loss of receptor function (Lerner et al., 1996). For PAR_1, a short region of the NT (residues 83–93), in addition to an acidic residue on extracellular loop 3, have been demonstrated to play a role in PAR-AP activation (Bahou et al., 1994; Blackhart et al., 2000). Intracellular loop 2 (ICL2) has been proposed to play a role in G-protein coupling for PAR_1 (Verrall et al., 1997), whereas the cytoplasmic tail contains phosphorylation sites that are involved in signal termination and internalization of the receptor (Trejo and Coughlin, 1999; DeFea et al., 2000). PARs possess potential N-linked glycosylation sequons (61) on their NT and ECL2. Not all potential sites of glycosylation are shown. In PAR_2, NT glycosylation has been demonstrated to regulate the ability of mast cell tryptase to activate the receptor (Compton et al., 2001).

has been heralded by the discovery of a dysfunctional polymorphic form of human PAR_2 with a phenylalanine to serine mutation at position 240 of loop 2 (Compton et al., 2000). This polymorphic receptor exhibits a reduced sensitivity to trypsin activation and an altered responsiveness to PAR-APs (Compton et al., 2000). In summary, much evidence points to a key role for extracellular loop 2 of PAR_1 and PAR_2 (and by extension, probably PAR_4) in the action of synthetic receptor-activating peptides. Whether the structural determinants for activation of this interesting extracellular domain of the PARs by synthetic peptides will be the same for activation of the receptor by the proteolytically revealed tethered ligand remains to be explored in depth. This aspect of PAR function, which is unique in comparison to other G-protein-coupled receptors, is an area that merits an extensive evaluation in the future.

D. Signaling, Desensitization, and Receptor Internalization

Once activated by their tethered ligands, the PARs produce a transient signal [e.g., a pulse of elevated intracellular calcium lasting less than 2 min or a transient effect on vascular tension (contraction or relaxation) lasting from 5 to 10 min]. This type of transient response elicited by the PARs differs from responses triggered by many other G-protein-coupled receptor systems, such as the sustained elevation of vascular tension caused by phenylephrine acting via the α-adrenoreceptor. Since, unlike other G-protein-coupled receptors, PARs are continuously exposed to the tethered ligand that cannot diffuse away, these questions arise: 1) how is the receptor rapidly desensitized? and 2) how does the cell recover its cell-surface complement of intact PARs to respond again efficiently to proteinases over time? The answer to these questions, at least insofar as PAR_1 and PAR_2 are concerned (and by extension one may include PAR_4), resides in the sequences of the intracellular receptor domains responsible for G-protein coupling (Verrall et al., 1997), receptor desensitization (Ishii et al., 1994; DeFea et al., 2000), and receptor internalization (Dery et al., 1999; Trejo and Coughlin, 1999; DeFea et al., 2000). All three of the PARs that activate an intracellular signal (PAR_1, PAR_2, and PAR_4) can cause an elevation of intracellular calcium via a presumed $G_{q/11}$-mediated process. For PAR_1, a role for intracellular loop 2 has been shown for G_q coupling (Verrall et al., 1997). PAR_1 (but not PAR_4; Faruqi et al., 2000) can couple not only to the G_q family, but also to the pertussis toxin-sensitive G_i (Hung et al., 1992; Swift et al., 2000) resulting in: 1) an inhibition of adenylyl cyclase and 2) presumably other processes, such as ion channel regulation and phospholipase Cβ and cSrc activation triggered by the $G_{i\alpha}/G_{\beta\gamma}$ subunits (reviewed by Macfarlane et al., 2001). The coupling of PAR_1 to G_q, G_i, or both very likely depends on the cell type and the relative abundance of the two G-proteins. Evidence also suggests that PAR_1 can couple to members of the $G_{12/13}$ family (Offermanns et al., 1994). One difficulty in interpreting some of the data in the literature obtained before the discovery of PAR_4 is that thrombin, which can activate both PAR_1 and PAR_4 (e.g., in human platelets, where both receptors are present), was often used as an agonist rather than the receptor-selective PAR-APs. Thus, although it is clear that both PAR_1 and PAR_4 can elevate intracellular calcium, the coupling of the different PARs to other specific members of the G-protein family remains to be established unequivocally. It should be noted further that PAR_1 and PAR_4 can exhibit distinct signaling shut-off and internalization kinetics, due to distinct potential phosphorylation sites in the C-terminal domains of the two receptors (see below and Shapiro et al., 2000). Thus, the kinetics as well as the G-protein-coupling specificity among PAR_1, PAR_2, and PAR_4 will very likely be found to differ. Many of the downstream signal pathway targets that can be activated, including members of the extracellular signal-regulated kinase/mitogen-activated protein kinase/stress-activated protein kinase/c-Jun NH_2-terminal kinase family, Src-family, other tyrosine kinases, and phosphatidylinositol-3 kinase, have been well summarized in a recent review (Macfarlane et al., 2001).

The signal termination and internalization processes have so far been studied primarily for human PAR_1 and PAR_2. For both PAR_1 and PAR_2, site-targeted phosphorylation in the receptor C-terminal domain by either the G-protein-coupled receptor kinases (Krupnick and Benovic, 1998) for PAR_1 (Ishii et al., 1994; Shapiro et al., 1996; Hammes et al., 1999; reviewed by Macfarlane et al., 2001) or protein kinase C for PAR_2 (Böhm et al., 1996a), involving subsequent interactions with β-arrestin and dynamin (Dery et al., 1999; DeFea et al., 2000) plays a key role for both signal down-regulation/desensitization and receptor internalization.

The characteristics of PAR trafficking [movement from an intracellular store (in the Golgi) to the plasma membrane, and internalization and recycling] differ in a number of unique respects from the dynamics of other members of the G-protein-coupled receptor family. Unlike the β-adrenoreceptor, PAR_1 in selected cell types appears to have an intracellular reservoir from which the cell-surface receptor can be efficiently replenished via a mechanism regulated by the signaling/internalization process itself (Hein et al., 1994). This "reservoir" of PAR_1 does not appear to be present in all cell types (e.g., platelets; discussed by O'Brien et al., 2001). Furthermore, rather than being recycled to the plasma membrane after activation and internalization, as is the G-protein-coupled receptor for substance P, PAR_1 is selectively targeted to the lysosome (Trejo and Coughlin, 1999). Notwithstanding, in some cell environments, a proportion of PAR_1, cleaved and activated by thrombin, appears to become available again at the cell surface in order to still be sensitive to activation by the PAR_1AP, SFLLRN-amide (Hoxie et al., 1993; Hammes and Coughlin, 1999). These data suggest that following activation of PAR_1, the exposed tethered ligand can subse-

quently become sequestered from activating the receptor further, but the receptor at the cell surface is not desensitized to PAR_1AP activation. Such a mechanism would yet in another way distinguish PAR_1, and very likely, other active members of the PAR family, from most of the other G-protein-coupled receptor families known to date. The subtle distinctions for the desensitization/internalization and trafficking properties among PARs 1, 2, and 4 remain to be determined in detail.

E. Receptor Activation and Proteinase Susceptibility: What Are the Endogenous Proteinase-Activated Receptor Regulators?

Because of its unique "hirudin-like" thrombin-binding domain downstream of the cleavage/activation site, PAR_1 is unusually sensitive to thrombin activation compared with PAR_4. Lacking a key proline residue at the P_2 position of the cleavage/activation site, PAR_2 is not activated by thrombin at all. Nonetheless, trypsin, depending on its concentration, is able to activate signaling by all of PAR_1, PAR_2, and PAR_4, indicating susceptibility to serine proteinase cleavage at the R/S (PAR_1 and PAR_2) or G/S (PAR_4) tethered ligand activation site in the three PARs. Since there are also other basic (Lys and Arg) amino acid residues carboxy-terminal to the cleavage/activation sites of the PARs, it is not surprising that other serine proteinases can either activate [e.g., cathepsin G activation of PAR_4 (Sambrano et al., 2000); streptokinase-plasminogen activation of PAR_1 (McRedmond et al., 2000); and granzyme A activation of PAR_1/PAR_4 (Suidan et al., 1994)] or disarm the PARs by cleaving the tethered ligand domain from the receptor [e.g., cathepsin G and plasmin disarming of PAR_1 (Kuliopulos et al., 1999; Molino et al., 1995)]. In this regard, trypsin has been found both to activate and disarm PAR_1 (Kawabata et al., 1999), and a small activation of PAR_1 by plasmin can be observed (Vouret-Craviari et al., 1995; Ishihara et al., 1997) as opposed to the main disarming action of plasmin on PAR_1 (above and Kimura et al., 1996). Conceptually, the proteinases, depending on their PAR cleavage targets, can act as either functional agonists or antagonists. A further level of regulation can occur in the setting of proteinase-inhibitor production (e.g., the serpins). Therefore, an additional singular feature of the PARs, among the G-protein-coupled receptor superfamily, is that the PARs can be seen as having their own endogenous set of functional agonists and antagonists. Like PAR_1, PAR_2 can be activated by serine proteinases other than trypsin [e.g., tryptase (Molino et al., 1997), mite-derived proteinases (der p3/p9; Sun et al., 2001), and factors Xa/VIIa (Bono et al., 2000; Camerer et al., 2000)]. In principle, PAR_2, like PAR_1, could also be silenced by cleavage at a point downstream from its tethered ligand (Loew et al., 2000). The restricted activation of PAR_2 by human tryptase, which cannot fully activate glycosylated PAR_2 as does trypsin (Compton et al., 2001), illustrates a novel glycosylation-dependent "control mechanism" for receptor activation that has not been observed for members of other G-protein-coupled receptor families. Whether or not glycosylation of the N-terminal domains of PAR_1 and PAR_4 will alter their susceptibility to selective activation or silencing by other proteinases remains to be determined.

For PAR_1 and PAR_4, it is clear that thrombin can play a key role as the "physiological" receptor activator. In the digestive tract, trypsin is present at concentrations sufficient for it to act as an endogenous PAR activator, depending on the local abundance of tissue-derived trypsin inhibitors (Kong et al., 1997). Furthermore, mast cell tryptase has been suggested as a potential endogenous activator of PAR_2 in humans, although the susceptibility of PAR_2 to tryptase activation in vivo may be restricted. That said, it is known that other members of the trypsin family capable of activating PAR_2 can be detected as proenzymes in a variety of settings [e.g., endothelial trypsinogen 2 (Koshikawa et al., 1997) that could potentially activate PAR_2 (Alm et al., 2000) and a cell-surface serine proteinase termed matriptase (Oberst et al., 2001) or membrane-type serine proteinase-1 (Takeuchi et al., 2000) that can also activate PAR_2]. Thus, as opposed to classical G-protein-coupled receptors that are activated by a select number of endogenous hormones or neurotransmitters, the PARs may be subject to both activation and inhibition (i.e., disarming) by a variety of proteinases, quite apart from the key enzymes (thrombin, trypsins, and tryptase) that are presently considered to be endogenous PAR regulators (see Table 1).

V. Physiological Roles of Proteinase-Activated Receptors

A. Thrombin Targets: Proteinase-Activated Receptors 1, 3, and 4

Quite apart from the its function in the coagulation cascade, the actions of thrombin on a variety of target cells are recognized as key factors in the hemostatic and inflammatory responses to injury. Thus, many of the physiological functions of thrombin are due in large part to the actions of thrombin on platelets, endothelial cells, and leukocytes (Cirino et al., 2000; summarized by Coughlin, 2000; Macfarlane et al., 2001). Many, if not most, of these cellular responses may result from the activation of one or both PAR_1 and PAR_4. However, as pointed out above, thrombin can potentially affect cells via a noncatalytic mechanism involving its chemotactic/mitogenic peptide domains; and thrombin can also activate enzymes, such as pro-matrix metalloproteinase-2 (Lafleur et al., 2001), to affect cell behavior. Furthermore, thrombin can modulate platelet function via a glycoprotein Ib-mediated signaling mechanism that appears to act synergistically with PAR_1 and PAR_4 (Soslau et al., 2001). To distinguish between the ability of thrombin to act via the PARs as opposed to other mechanisms, it has been of enormous value 1) to use PAR-specific

agonists (e.g., TFLLR-NH_2 for PAR_1, SLIGRL-NH_2 for PAR_2, or AYPGKF-NH_2 for PAR_4); 2) to evaluate responses in PAR-deficient mice; and 3) to employ selective PAR antagonists that do not exhibit intrinsic activity (Andrade-Gordon et al., 1999; Ahn et al., 2000; Hollenberg and Saifeddine, 2001). This same kind of approach has yielded much information about the potential physiological role(s) that may be subserved by PAR_2 (see below), although a PAR_2 antagonist is not yet available. Stemming from such studies, it is clear that, as anticipated, PAR_1 plays an aggretory and secretory role in human platelets and a role as a regulator of endothelial cell function. What might not have been predicted is that PAR_1 can also play an important role in embryonic development via its impact on endothelial cell function (Griffin et al., 2001) and in the process of restenosis after vascular injury (Andrade-Gordon et al., 2001), very likely due to PAR_1-mediated actions on vascular endothelial and smooth muscle cells. The widespread tissue distribution of PAR_1, ranging from the vasculature to the brain, lung, and gastrointestinal tract (Table 3), predicts a multifactorial role for PAR_1, including neurogenic inflammatory responses as well as cardiovascular effects (Cocks and Moffatt, 2000; de Garavilla et al., 2001; summarized by Macfarlane et al., 2001; Vergnolle et al., 2001b). That PAR_1 has been found to be an "oncoprotein" in the sense of conferring a "transformed" phenotype on NIH 3T3 cells (focus formation and anchorage- and serum-independent growth; Martin et al., 2001) and in its ability to modulate cellular invasion (Even-Ram et al., 1998, 2001; Henrikson et al., 1999; Kamath et al., 2001) adds another dimension to the potential roles that PAR_1 may play in the setting of pathophysiology.

The physiological actions of thrombin that are due to PAR_4 activation have yet to be clarified, except as a key for thrombin-mediated aggregation of human and rodent platelets (Kahn et al., 1998, 1999). Experiments with both platelets from PAR_4 −/− mice (Sambrano et al., 2001) and a PAR_4 antagonist in a rat platelet preparation (Hollenberg and Saifeddine, 2001) have unequivocally established this essential role for PAR_4 in the activation of murine and rat platelets by thrombin. That cathepsin G and trypsin (and presumably, other members of the trypsin family) can also efficiently activate PAR_4 indicates that the pathophysiology of PAR_4 may well be broader than the effects of thrombin indicate. Messenger RNA for human PAR_4 has been detected in a variety of tissues, with comparatively higher amounts present in human small intestine, liver pancreas, lung, thyroid, and testis, and smaller amounts of message detected in a number of human organs, including the prostate, adrenal gland, and trachea (Xu et al., 1998; see also Table 3). The tissue distribution of PAR_4 mRNA in the mouse appears to differ from that in the human, with larger amounts found in spleen and bone marrow but relatively low expression in liver or testis (Kahn et al., 1998). There may, therefore, be marked differences between species in the distribution of PAR_4, which will need to be taken into consideration when exploring the pharmacology of the PAR_4APs in different animals and generalizing findings obtained from one species to another. To date, the expression of PAR_4 in different tissues has not been compared with using an immunohistochemical approach. Thus, apart from its function in platelets, the diverse physiological role that PAR_4 may play represents a most interesting topic for further investigation.

The ability of thrombin to cleave PAR_3 with high efficiency and potency because of the putative interaction of the anion exosite binding domain of thrombin with the negatively charged postcleavage sequence in PAR_3 presents a puzzle, since on its own, murine PAR_3 appears not to generate an intracellular signal but rather acts as a cofactor for PAR_4 activation (Nakanishi-Matsui et al., 2000; Sambrano et al., 2001). It will be of interest to determine whether the lack of PAR_3 signaling can be attributed to special features of its intracellular domains (e.g., see Fig. 2). The wide tissue distribution (Table 3) of PAR_3 mRNA in humans, with large amounts in stomach, small intestine, and trachea, as well as in bone marrow, along with the more restricted distribution in murine tissues (splenic megakaryocytes) (Ishihara et al., 1997), presents a puzzle in terms of what the potential physiological role for PAR_3 may be.

B. Proteinase-Activated Receptor 2, a Trypsin Target

Like PAR_1, mRNA for PAR_2 can be detected in quite a number of tissues (Table 3), with strong signals observed in the gastrointestinal tract (stomach, small intestine, and colon) as well as in the kidney, with a comparable distribution in murine and human tissues (Nystedt et al., 1994; Böhm et al., 1996b). The availability of antisera that react with PAR_2 has confirmed the presence of the receptor in a large number of tissues, in parallel with the detection of receptor mRNA and, in addition, has served to localize the receptor on enteric nerves (D'Andrea et al., 1998; Corvera et al., 1999). In the absence of effective PAR_2 antagonists, it has been the use of receptor-selective PAR_2APs (e.g., SLIGRL-NH_2), along with experiments done with PAR_2 −/− mice, that has provided insight as to the potential physiological role the receptor may play. To date, although the PAR_2-deficient mouse does not exhibit a distinct phenotype, the actions of the PAR_2APs suggest roles in the cardiovascular, pulmonary, and gastrointestinal systems (summarized in reviews by Cocks and Moffatt, 2000; Macfarlane et al., 2001; Vergnolle et al., 2001b), with an added intriguing impact in the setting of inflammation and nociception that involves the activation of PAR_2 on sensory nerves (Steinhoff et al., 2000; Vergnolle et al., 2001a). Evidence for a role for PAR_1 in triggering neurogenic inflammation has also been obtained (de Garavilla et al., 2001). If there is an overriding principle

that can be put forward for the physiological function of PAR_1, PAR_2, and PAR_4, one can point to their potential role in the settings of tissue injury, repair, and remodeling, including processes that take place during embryo development. In such a context, locally produced tissue proteinases, including thrombin, other enzymes in the coagulation and complement cascades, trypsin(ogens), as well as many other proteinases will undoubtedly be found to exert a key impact on tissue signaling via the PARs.

VI. Future Issues and Conclusions

With insulin and adrenaline, both known as hormones since the early part of the 20th century, knowledge of their potential physiological roles was consolidated long before their receptor mechanisms were elucidated. However, for the PARs, it would appear that the story is playing itself out in the reverse. That is, it is clear that for PAR_1, PAR_2, and PAR_4 variations on the G-protein-coupled receptor paradigm are responsible for their mechanisms of action, but the precise physiological role(s) that these receptors play, apart from the hemostatic/inflammatory/repair processes triggered by thrombin, are largely unknown. This is the more so because all of the PARs can be targets for a variety of proteolytic enzymes that can either act as surrogate receptor activators or as receptor "silencers". Thus, in a sense, the cadre of endogenous proteinases that can function as PAR regulators represents a "degenerate" agonist/antagonist "code" that sets this receptor family apart from other G-protein-coupled receptors that have a clear-cut set of endogenous agonists and few, if any, endogenous antagonists. This distinct PAR receptor paradigm is one issue that represents an intriguing challenge for future work with the PARs aimed at understanding their physiology. To this end, the use of selective PAR-targeted agonists and the development of potent PAR-targeted antagonists for all the PARs will play a key role, as will the use of PAR $-/-$ animals. It is difficult to predict the degree of "backup redundancy" that will exist for the physiological actions of the PARs, but the data available so far (e.g., the key role of endothelial PAR_1 for vascular development as opposed to PAR_2, which is also present in the endothelium) suggest that each of the PARs will be found to play a distinct physiological role. The challenge to elucidate the potential role of PAR_3 further is even more difficult since murine PAR_3 receptor, upon proteolytic cleavage, does not on its own generate an intracellular calcium signal. Furthermore, the data obtained to date also indicate that, despite similarities in G-protein coupling, quite distinct signal transduction pathways will be found to subserve each of PAR_1, PAR_2, and PAR_4. This situation is already clear in the case of the distinction between PAR_1 and PAR_4, for which one receptor (PAR_1) can signal dually via both G_q and G_i, whereas in the same cellular context, the other receptor (PAR_4) appears unable to signal via G_i, although it does couple to G_q (Faruqi et al., 2000). Thus, another avenue that merits further exploration relates to the distinct signaling, desensitization, internalization/degradation, and recycling/replenishment processes that may occur for each of the distinct PARs. Ultimately, a most difficult question to resolve may be: what, indeed, are the endogenous PAR-regulating proteinases, and in which settings might such proteinases play a physiological role? This is a unique question, in relation to the PARs, that does not readily apply to other G-protein-coupled receptors.

Finally, there is the question as to how many more PARs there may be. Given that, to date, an extensive database search has resulted in the recent discovery of only one more member of the family (PAR_4), it may well be that other family members, if they exist, will have quite distinct sequences, even at the extracellular loop 2 locale, wherein the tripeptide sequence CHD has been found to be present in all PARs so far discovered. Alternatively, even though PAR_4 is situated on a chromosome (human 19p12; mouse 8B3.3) distinct from that of PAR_1, PAR_2, and PAR_3 (human 5q13; mouse 13D2), the presumed gene duplication giving rise to three family members tightly clustered on one chromosome may not have occurred at the site of the PAR_4 gene. Thus, at this stage in the course of PAR discovery, the number of PARs is limited to four. Notwithstanding, data obtained with the PAR-APs, using the classical pharmacological approach pioneered by Ahlquist (1948), suggest that there may be more receptors present in several tissues (endothelium and gastrointestinal tract; Roy et al., 1998; Vergnolle et al., 1998) than can be accounted for by the PARs cloned so far. As mentioned already, the receptors for these peptides may or may not turn out to be activated by proteinases. On the other hand, it is clear that a number of well known receptors (e.g., the one for insulin) can be subject to proteolytic activation, and it is quite likely that receptors yet to be discovered will be found to be targets for proteolytic activation/silencing by proteinases other than those belonging to the serine proteinase family.

In conclusion, the search for the receptor responsible for the actions of thrombin has led in a most unexpected direction, resulting in the discovery of a novel G-protein-coupled receptor family that now has four family members. The distinct mechanism of activation, via the proteolytic unmasking of a set of distinct tethered ligands for each of the PARs, clearly sets this family of receptors apart from all other G-protein-coupled receptors discovered so far. The pharmacodynamics of the PARs, including their "irreversible" mechanism of activation via a tethered ligand agonist unmasked by a triggering proteinase, have led to a shift in the conventional paradigm used to deal with agonist/receptor interactions and have put proteinases into the spotlight as signaling molecules rather than simply as enzymes responsible for enteric

digestion and remodeling extracellular matrix. The nomenclature adopted to designate this receptor family underscores the connection between the receptor family and proteinases and prompts a number of challenging future directions to determine the physiological roles these receptors and their regulatory proteinases will play. For the PARs, it can be said that the story is just at its beginning and that there are many more intriguing chapters to be written.

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