Protease-Activated Receptors in Neuronal Development, Neurodegeneration, and Neuroprotection: Thrombin as Signaling Molecule in the Brain

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Protease-activated receptors (PARs) belong to the superfamily of seven transmembrane domain G protein-coupled receptors. Four PAR subtypes are known, PAR-1 to -4. PARs are highly homologous between the species and are expressed in a wide variety of tissues and cell types. Of particular interest is the role which these receptors play in the brain, with regard to neuroprotection or degeneration under pathological conditions. The main agonist of PARs is thrombin, a multifunctional serine protease, known to be present not only in blood plasma but also in the brain. PARs possess an irreversible activation mechanism. Binding of agonist and subsequent cleavage of the extracellular N-terminus of the receptor results in exposure of a so-called tethered ligand domain, which then binds to extracellular loop 2 of the receptor leading to receptor activation. PARs exhibit an extensive expression pattern in both the central and the peripheral nervous system. PARs participate in several mechanisms important for normal cellular functioning and during critical situations involving cellular survival and death. In the last few years, research on Alzheimer's disease and stroke has linked PARs to the pathophysiology of these neurodegenerative disorders. Actions of thrombin are concentration-dependent, and therefore, depending on cellular function and environment, serve as a double-edged sword. Thrombin can be neuroprotective during stress conditions, whereas under normal conditions high concentrations of thrombin are toxic to cells. NEUROSCIENTIST 10(6):501–512, 2004. DOI: 10.1177/1073858404269955

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Thrombin, Protease-Activated Receptors (PARs), and Distribution of PARs in the Brain

Four subtypes of PARs are known. They are called PAR-1 to PAR-4. The main agonist of PAR-1, PAR-3, and PAR-4 is thrombin, whereas PAR-2 is activated by trypsin, an intestinal digestive enzyme. During the blood coagulation cascade, thrombin, a 39 kDa active and functional protein, is generated through cleavage of prothrombin, a 71.6 kDa precursor molecule by factor Xa. Prothrombin mRNA is known to be expressed in several regions of the CNS, such as the olfactory bulb, cortex, cerebellum, hippocampus, and thalamus. The biochemical mechanism of PAR activation involves that besides the protease also short synthetic peptides known as "activating peptides" can activate PARs.

Several studies from our laboratory have shown the presence of all four PAR subtypes in the brain (Striggow and others 2001; Wang, Ubl, and Reiser 2002). Immunohistochemical analysis of PAR-1 to -4 presence in the brain showed that PAR-1 protein occurs abundantly in the hippocampus (pyramidal cell layer) compared with low expression levels found in the cortex, thalamus, hypothalamus, striatum, and amygdala neurons (Striggow and others 2001). Both mRNA and PAR-1 protein have been reported in the embryonic and postnatal brain (Niclou and others 1998; Weinstein and others 1995).

PAR-2 and PAR-3 proteins are widely detected in the hippocampus, cortex, amygdala, thalamus, hypothalamus, and striatum (Striggow and others 2001). PAR-2 protein is also present in the CNS during embryogenesis (Jenkins and others 2000). PAR-4 protein is found in the neuronal soma, axons, and dendrites of the hippocampus, all cortical layers, thalamus, hypothalamus, and amygdala (Striggow and others 2001). Furthermore, we have seen functional coexpression of PAR-1 to -4 in cultured rat astrocytes (Ubl and others 1998; Wang, Ubl,

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and Reiser 2002). Presence of all 4 PARs in cultured rat astrocytes was verified at transcription (mRNA), translation (protein), and functional levels. The functional state of the receptors was confirmed by calcium mobilization assay. Using reverse transcription-polymerase chain reaction (RT-PCR) and immunostaining, we have recently detected expression of PAR-1 and PAR-3 both in cultured rat oligodendrocytes and in an oligodendrocyte cell line (Wang and others 2004). Although the oligodendrocyte cell line functionally shows PAR-1 as observed by transient calcium increase due to release of calcium from intracellular stores upon agonist stimulation, PAR-3 exhibits no apparent functional activity.

PARs are highly expressed in other regions of the nervous system as well. In the spinal cord, PAR-1 mRNA is abundant in motor neurons, in the dorsal root ganglia, and in preganglionic neurons of the autonomic nervous system (Niclou and others 1998). PAR-2 protein is also detected in the peripheral nervous system (PNS) during embryogenesis (Jenkins and others 2000). PAR-2 is expressed by primary spinal afferent neurons (Steinhoff and others 2000). Using immunocytochemistry, recently PAR-4 expression has been found in peripheral nerves (D'Andrea and others 2003). Apart from the nervous system, PARs are highly enriched in a variety of cells and tissues (Böhm, Kong, and others 1996; D'Andrea and others 1998; Ishihara and others 1997; Rohatgi and others 2003; Xu and others 1998).

Thrombin and PARs in the Brain under Physiological and Pathophysiological Conditions

A glance over the vast expanse of work done on PARs indicates the importance of these receptors in a variety of cellular functions in different tissues, especially in the brain. Recent studies involving thrombin and PARs in the nervous system have also shown that this family of receptors is actually involved in varied functions in the nervous system and plays a very critical part in maintaining a delicate balance between neuroprotection and neurodegeneration during inflammation, injury, and disease states.

Thrombin is truly multifunctional. Even in the nervous system it can support growth, maintenance, and morphological changes. For instance, it elicits cell shape change in neurons. Thrombin activity results in the retraction of neurite processes, which is very specific and is known to be reversed upon thrombin removal from the tissue culture medium (Festoff and others 1996). Moreover, this protease activity was found to be mediated via PAR-1 (Jalink and Moolenaar 1992; Pai and Cunningham 2002). A recent study using a transgenic mouse for prothrombin showed that formation of thrombin from prothrombin in the nervous system seems to require blood-derived factors (Sinnreich and others 2004).

Astrocytes respond to thrombin in several ways. Thrombin leads to reversal of astrocyte stellate morphology in culture and makes them flat and epithelial in shape, while also inducing a mitogenic effect by increasing astrocyte cell number, as seen in culture (Cavanaugh and others 1990). However, both these effects of thrombin observed in astrocytes are mutually exclusive from each other in terms of the threshold concentration of thrombin required. No mitogenic effect has been observed at the lowest thrombin concentration, which causes astrocyte stellation reversal. Studies done in our laboratory have shown that application of thrombin and PAR-1– to PAR-3–activating peptides results in astrocyte proliferation (mediated via PARs), whereas PAR-4–activating peptide has either no effect on cell number in case of short incubation or causes reduction in cell number with prolonged incubation (Wang, Ubl, and Reiser 2002). Furthermore, thrombin stimulates release of a potent vasoconstrictor peptide, endothelin-1, from astrocytes (Ehrenreich and others 1993) and induces expression of the proto-oncogenes c-fos and c-jun mRNA in astrocytic cell line (Trejo and others 1992). Thrombin also stimulates release of arachidonic acid (AA) by astrocytes, which in its turn suppresses thrombin-evoked-Ca^{2+} response in astrocytes (Sergeeva and others 2002).

Apart from astrocytes, thrombin via PAR-1 activation induces proliferation also of microglia (Suo and others 2002). Microglia cells are resident macrophages in the CNS and play a significant role in inflammation and neuronal cell death in CNS disorders. PAR-1 activation in microglia up-regulates CD40 expression, which is a transmembrane glycoprotein with limited homology to tumor necrosis factor receptor (TNF-R) and is expressed on immune cells. This also potentiates CD40 ligand-induced TNF-α production in microglia and thus leads to microglia activation (Suo and others 2002). TNF-α is a pro-inflammatory cytokine involved in CNS inflammation.

Thrombin not only activates PARs, a G protein-coupled receptor (GPCR), but this activation can also affect the expression of another type of GPCR, the metabotropic glutamate receptor, which works in different ways in synaptic plasticity. PAR-1 activation by thrombin and PAR-1–activating peptide reduces the expression of metabotropic glutamate receptor mGluR5 on astrocytes, which is seen both at mRNA and protein levels (Miller and others 1996). Astroglia are major components of glutamatergic pathways and have important roles in maintaining glutamatergic transmission. Knowledge about the regulation of astrocyte functions during pathological states connected with increased thrombin levels can be important in understanding neuronal transmission and interaction involved in CNS response to injury (Miller and others 1996). Proteolytic action of thrombin via PAR-1 in hippocampal neurons inhibits *N*-methyl-D-aspartate (NMDA) receptor-mediated pain processing by a pathway involving the endothelin-A receptor, which is also a GPCR (Fang and others 2003). Physiological functions of PAR-2 have been studied in detail in the gastrointestinal tract, in air-

way relaxation, and in skin development, whereas those of PAR-3 and PAR-4 are known in the case of platelet activation.

One area where thrombin and PARs have moved to the forefront of research is their potential role in the regulation of inflammation and the response to injury (Vergnolle and others 2001; Xi and others 2003). PAR-1 was found to be down-regulated after facial nerve injury (Niclou and others 1998). A recent study from our laboratory has shown that mild optic nerve crush in rat, which represents a mild trauma model of CNS, leads to transient up-regulation of all PARs (Rohatgi and others 2003). Using optic nerve crush in the rat, it has been observed that thrombin is produced at the site of tissue injury by injury-induced activation of prothrombin, which then triggers secondary damage (Friedmann and others 2001).

Surprisingly, thrombin via its receptor-mediated proteolytic action can induce contrasting effects, either apoptotic cell death in neural cells (Donovan and others 1997; Turgeon and others 1998) or neuroprotection (Donovan and Cunningham 1998), which has been seen both in astrocytes and neurons in culture. Opposite effects of thrombin observed in isolated cells prompted several studies to verify and establish whether there is a concentration-dependent action of thrombin. This was investigated particularly with regard to tissue repair and wound healing, as reported in the case of several brain injury models, like oxygen-glucose deprivation in hippocampal organotypic slices (Striggow and others 2000), optic nerve crush in the rat (Friedmann and others 1999; Friedmann and others 2001), and hypoglycemia or oxidative stress condition (Cunningham and Donovan 1997; Vaughan and others 1995). A study from our laboratory was able to prove that thrombin at a concentration of 50 nM or less can be neuroprotective, whereas at higher concentrations a neurodegenerative effect is elicited in an in vitro organotypic hippocampal slice culture model (Striggow and others 2000). The concomitant in vivo work in gerbils showed that injection of a specific thrombin inhibitor, the leech anticoagulant hirudin, before occlusion of both common carotid arteries to induce transient global cerebral ischemia leads to an increased neuronal survival. This signifies partial protection against a degenerative effect of endogenous thrombin (Striggow and others 2000).

A further study, which highlighted the opposite effects of thrombin, revealed that thrombin-mediated PAR-1 activation protects neurons and astrocytes against insults like oxidative stress or hypoglycemia, whereas high concentrations of thrombin are toxic to both neurons and astrocytes cultured under normal conditions (Vaughan and others 1995). The study further made clear that all these effects can be blocked by protease nexin-1 (PN-1, a cell surface as well as extracellular serine protease inhibitor), thus indicating the involvement of thrombin's proteolytic activity via PARs. Most of the effects of thrombin by proteolytic activity are brought about by its main receptor, PAR-1 (Chambers and others 1998; Weinstein and others 1998), but another study (Vergnolle and others 2002) has highlighted the role of PAR-4 receptor activation in proinflammatory properties of thrombin. Recent evidence indicates that proinflammatory effects of thrombin on brain microglia are induced not only by PAR-1 but also via PAR-4 (Suo, Wu, Citron, Gao, and others 2003). PAR-4 activation causes the detrimental effects of thrombin on microglia (Suo, Wu, Citron, Gao, and others 2003). PAR-2 is the most studied receptor subtype in terms of its role in inflammation in other tissues (Damiano and others 1999; Knight and others 2001). Agonists of PAR-2 are involved in protease-induced neurogenic inflammation (Steinhoff and others 2000). PAR-2 agonist is toxic to hippocampal neurons in a concentration-dependent manner, indicating that PAR-2 activation may contribute to neurodegeneration (Smith-Swintosky and others 1997).

A major neurodegenerative disorder where thrombin seems to play an important role is Parkinson's disease. In Parkinson's disease, a decrease in the number of neurons that release the neurotransmitter dopamine results in loss of coordinated movement and stiffness of limbs. On several occasions, it has been reported that thrombin-mediated activation of microglia is involved in neuropathological processes of dopaminergic neuronal cell death in rat substantia nigra (SN), which is associated with mechanisms of Parkinson's disease (Carreno-Muller and others 2003; Choi, Joe, and others 2003). Thrombin injection in vivo in SN induces microglia activation and results in selective damage of dopaminergic neurons. However, the degenerative effect of thrombin on dopaminergic neurons cannot be mimicked by thrombin receptor-activating peptide, which suggests that thrombin-induced nigral dopaminergic neurodegeneration is not mediated via PAR-1 (Choi, Lee, and others 2003).

PARs have been seen in connection also with several other neurodegenerative diseases, like Alzheimer's disease. To make major progress in the field of Alzheimer's disease, it is a prerequisite to understand the biology of β-amyloid protein, a causative agent of Alzheimer's disease. Some reports indicate a role for thrombin in regulating the neuropathology of Alzheimer's disease, as thrombin attenuates induction by β-amyloid of both neuronal degeneration and inhibition of astrocyte stellation (Pike and others 1996). Thrombin-mediated effects can in fact be mimicked by PAR-1–activating peptide, and can also be blocked by two specific thrombin inhibitors, hirudin and PN-1 (Pike and others 1996). This indicates the involvement of PAR-1 activation in Alzheimer's disease.

A recent study has revealed that even nanomolar concentrations of thrombin are capable of inducing rapid tau hyperphosphorylation in mouse hippocampal neurons via PAR-1 and PAR-4, leading to apoptotic neuronal cell death (Suo, Wu, Citron, Palazzo, and others 2003). Disruption of the cytoskeleton is a key feature in the development of Alzheimer's disease pathology. Tau, which is one of the microtubule-associated proteins, occurs as an axonal phosphoprotein in the normal adult

Table 1. Protease-Activated Receptor (PAR) Functions in the Brain: Functional Consequences of Thrombin-Mediated PAR Activation in Different Cell Types of the Brain and Their Role in Neurodegenerative Disorders

Brain Cell Type	Consequences of PAR Activation
Neurons	Apoptosis; cytoprotection; cell process retraction; glutamate (*N*-methyl-D-aspartate) receptor potentiation
Astrocytes	Apoptosis; cell proliferation; cytoprotection; endothelin-1 release; increase in cytokine-induced nitric oxide (NO) release and iNOS (nitric oxide synthase) expression; loss of stellate morphology; reduced mRNA and protein levels of mGluR5
Microglia	NO production; potentiation of tumor necrosis factor-α production and release of other cytokines; cell proliferation
	Possible involvement of PARs in neurodegenerative disorders • Tau hyperphosphorylation and apoptotic neuronal cell death in Alzheimer's disease. • Increased PAR-1 expression in astrocytes in HIV encephalitis brains. • Role in neurogenic inflammation. • Decreased infarct volume after transient focal cerebral ischemia in PAR 1 –/– mouse. • Participation of endogenous thrombin in ischemic preconditioning.

brain. However, in Alzheimer's disease, tau is hyperphosphorylated, and apart from axons, this protein is also found in cell bodies and dendrites of the affected neurons. Hyperphosphorylated tau is the major constituent of paired helical filaments, which form the neurofibrillary tangles, which is one of the histopathological hallmarks of Alzheimer's disease. The number of neurofibrillary tangles is directly related to the degree of dementia seen in Alzheimer's patients and consequently seems to control neuronal dysfunction. Therefore, proteolytic activity of thrombin, via both PAR-1 and PAR-4, in the induction of tau hyperphosphorylation and the subsequent formation of neurofibrillary tangles highlights the importance of thrombin and PARs in the pathogenesis of Alzheimer's disease.

Lately, thrombin and PAR-1 have even been shown to be involved in human immunodeficiency virus–related neurodegeneration (Boven and others 2003). HIV encephalitis is the major cause of brain infection in HIV patients. Encephalitis is a type of brain inflammation of viral or other microbial origin. Both mRNA and protein levels of thrombin and PAR-1 were found to be significantly increased in astrocytes in HIV encephalitis brains (Boven and others 2003). Activation and up-regulation of PAR-1 seem to contribute to brain inflammation and neuronal damage during HIV-1 infection (Boven and others 2003).

Stroke is a sudden neurological deficit caused either by occlusion of cerebral blood vessels, leading to ischemic necrosis of the brain (cerebral infarction), or by rupture of blood vessels, resulting in hemorrhage in the brain or in the subarachnoid space (intracranial hemorrhage). Studies on PAR expression in different animal models of cerebral ischemia from our laboratory have shown that PARs might be involved in the pathophysiology of cerebral ischemia, in that PAR mRNA expression was shown to be transiently affected by transient focal cerebral ischemia (Rohatgi and others 2004; Striggow and others 2001). A threefold reduction in infarct volume has been found in PAR-1 knockout mice after transient focal cerebral ischemia, indicating a neurodegenerative role of PAR-1 during stroke and ischemia and an induction of neuroprotection due to lack of PAR-1 (Junge and others 2003). In contrast, neuroprotective actions of activated protein C, another serine protease, were found to be mediated via both PAR-1 and endothelial protein C receptor in a mouse model of focal cerebral ischemia (Griffin and others 2004).

Another aspect studied to underpin the importance of thrombin as a potential neuroprotective agent is its function in ischemic preconditioning. Preconditioning is a phenomenon whereby the brain develops tolerance to a lethal insult by pretreatment with a physiologically stressful but less severe insult. In that way, preconditioning provides a useful therapeutic window, especially to stroke patients. We have shown that endogenous thrombin is involved in ischemic preconditioning. Injection of hirudin before the induction of mild ischemic insults increased the damage after a subsequent severe ischemic insult (Striggow and others 2000). This demonstrates neuroprotection by thrombin. Preconditioning by application of thrombin in vivo also attenuates brain edema formation, which is caused by cerebral hemorrhage (Xi and others 1999). Table 1 provides an overview across some of the major functional consequences, that is, morphological, physiological, and biochemical, of PAR activation in all three main types of brain cells. Moreover, participation of PARs in several of the neurodegenerative disorders, which have been described in detail in the text, is summarized.

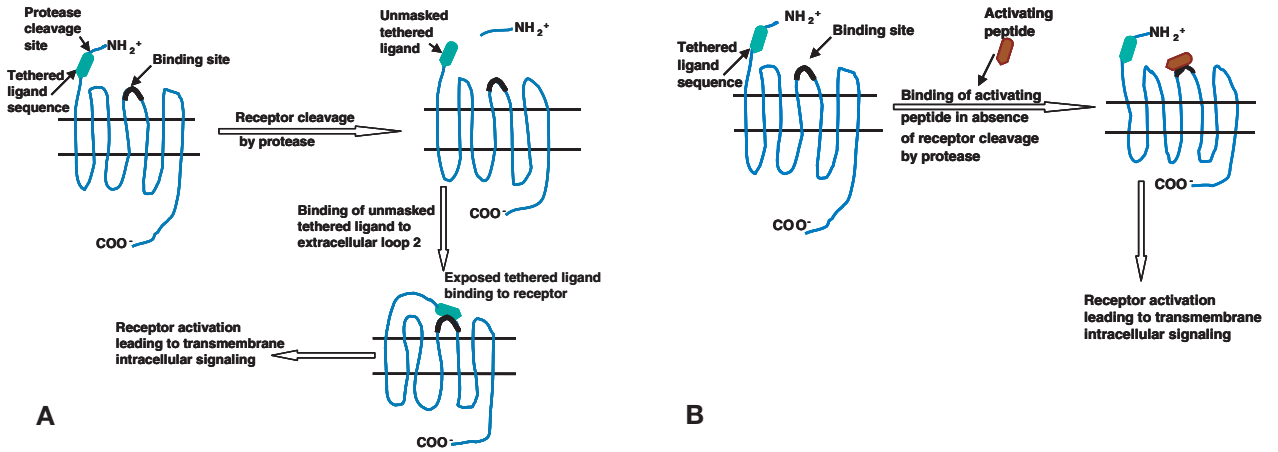

Fig. 1. PAR (protease-activated receptor) structure and activation mechanism: The receptor polypeptide (blue) is made up of an extracellular N-terminus (NH_2^+) followed by seven transmembrane domains and an intracellular C-terminus (COO^-). The transmembrane domains are connected by means of three extracellular and three intracellular loops. Proteolytic activation of the receptor by serine proteases at the N-terminus results in unmasking of the tethered ligand sequence (green pentagon structure), which then binds to a specific binding site (black) for the tethered ligand on extracellular loop 2 and causes receptor activation (*A*). In the absence of proteolytic cleavage by proteases, receptor activation can take place by binding of synthetic activating peptide (brown pentagon structure) corresponding to the tethered ligand sequence of the receptor directly to the binding site on extracellular loop 2 (*B*).

Proteases as Signaling Molecules Acting via PARs

Genome analysis revealed that among the enzymes, proteases form the largest group. In recent years, research on proteases has dramatically changed the concept of how we view this family of enzymes. Owing to their highly specific mode of action, that is, hydrolysis of peptide bonds in proteins, proteases regulate a wide variety of biological processes including several pathological processes. Proteases are even referred to as "signaling scissors." On the basis of their catalytic mechanism, proteases are divided into four classes. In the course of evolution, each of them has specialized to perform certain specific functions. These classes are named by the amino acid essentially involved in the catalytic mechanism: 1) serine proteases (examples: thrombin, trypsin, and chymotrypsin; this class is the focus of the overview presented here); 2) aspartic (example: pepsin family of digestive enzymes); 3) cysteine (examples: lysosomal cathepsin and cytosolic calpains); and 4) metalloproteinases, with metal ions essential for their proteolytic activity (example: thermolysin, a thermostable extracellular metalloendopeptidase containing four calcium ions).

Some proteases are secreted by cells into the surrounding tissue to cause destruction of proteins in the extracellular material. Alternatively, proteases act locally in the breakdown of a particular protein. The hydrolytic action of proteases is irreversible, and once a peptide bond is broken, the protein cannot be reverted to its original state. Hence, most of the proteases are expressed as proenzymes until their action is required. When proteolytic processing involves highly specific and limited substrate cleavage, it can control the intracellular and extracellular distribution of several proteins and their activity.

Cell signaling, which is important for the existence, maintenance, and continuation of homeostasis, provides a network between different cells with cascades of sequential signals. Serine proteases like thrombin and trypsin can also act as signaling molecules to bind and activate specific cell surface receptors, PARs, and in this way regulate multiple cellular functions. PARs are a family of transmembrane GPCRs that are associated inside the cell with proteins that bind guanine nucleotides and are therefore referred to as G proteins. PARs display the typical seven transmembrane domain helices, which are connected by extracellular and intracellular loops together with an extracellular N-terminal and intracellular C-terminal domain, as shown schematically in Figure 1(*A*).

PARs utilize a unique mechanism to convert an extracellular proteolytic cleavage event into an intracellular signal (Coughlin 2000). Figure 1(*A*) illustrates the mechanism of PAR activation in the presence of agonist proteases. The mechanism of receptor activation involves cleavage of the receptor at a specific site within the extracellular N-terminus owing to binding and proteolytic action of the agonist protease. The cleavage reveals a new N-terminal ligand tethered to the receptor polypeptide chain. PARs are in fact peptide receptors carrying their own innate ligand, which, when unmasked, binds intramolecularly to the receptor and thus activates transmembrane signaling. Only about six amino acid residues within the newly exposed tethered ligand interact with the binding site on extracellular loop 2 of the cleaved receptor (Coughlin 2000), as explained in Figure 1(*A*).

Trypsin is another serine protease that acts as an agonist of PARs. Trypsin is basically known as a digestive enzyme and occurs in the gastrointestinal tract as an inactive zymogen, trypsinogen. Apart from PAR-2, PAR-1 (Ubl and others 1998) and PAR-4 (Xu and others

1998) can also be activated by trypsin. Because trypsin is not present in the brain, studies were conducted to find PAR-2 agonists in the brain or other areas where trypsin has no access. Tryptase was found to activate PAR-2 with similar efficiency as PAR-2–activating peptide in cultured human endothelial cells (Molino and others 1997). Tryptase is a tetrameric serine protease released by mast cells after an allergic reaction.

Not only thrombin and trypsin but also some other serine proteases are also capable of activating PARs. Granzyme A, a serine protease released upon cytotoxic T lymphocyte stimulation, can activate PAR-1 in neurons and astrocytes (Suidan and others 1994). It is produced by cytotoxic T lymphocytes and under resting conditions resides in the secretory vesicles in cytoplasm. Granzyme A proteolytic activity results in both neurite retraction and reversal of astrocyte stellation. Serine proteases not only activate PARs, but some of them also inactivate them. Prominent among them are cathepsin G, elastase, proteinase 3, and plasmin. Metalloproteinase, such as thermolysin, can likewise inactivate PARs. The inhibitory proteases are released by neutrophils during inflammatory conditions. Their inactivation mechanism involves proteolytic destruction of the tethered ligand domain and thereby renders the receptor unresponsive to agonist proteases.

Apart from serine proteases, PARs can also be activated by short synthetic peptides known as "activating peptides," as shown in Figure 1(*B*). The amino acid sequence of the activating peptide corresponds to the tethered ligand sequence of the receptor and, as a result, can activate the receptor without the need for proteolytic cleavage. Figure 1(*B)* schematically represents the mechanism of PAR activation by synthetic activating peptides in the absence of agonist proteases. Hence, the receptor inactivated by an inhibitory serine protease can still be activated by the activating peptide. PAR-1–activating peptide derived from human PAR-1 sequence can be used to activate both PAR-1 and PAR-2 (Hollenberg and others 1997). Now a more specific PAR-1–activating peptide (TRag) is also known (Kawabata and others 1999; Wang, Ubl, and Reiser 2002). PAR-2–activating peptides, based on both human and mouse receptor sequences, are capable of activating PAR-2 and eliciting responses similar to trypsin, but they cannot activate PAR-1 (Hollenberg and others 1993). Recent studies from our laboratory have shown that PAR-3–activating peptide induces Ca^{2+} signaling in astrocytes, albeit to a very modest degree (Wang, Ubl, and Reiser 2002). PAR-4–activating peptide is capable of stimulating the receptor only at a high peptide concentration, and an increased incubation period leads to toxic effects in astrocytes (Wang, Ubl, and Reiser 2002).

Classification of PAR Subtypes and Mechanisms of PAR Signal Transduction

The PAR-1, PAR-2, and PAR-3 genes co-localize to the same region of the human genome on band number 13 of the long arm (q) of chromosome 5 (5q13), unlike

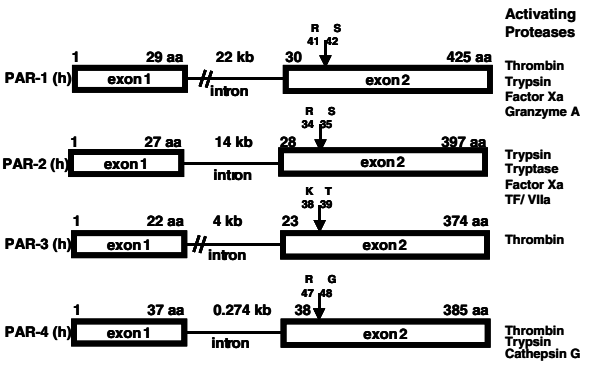

Fig. 2. Genomic organization of PAR (protease-activated receptor) genes: Exon-intron pattern of human (h) PAR-1 to -4 genes show presence of two exons for which the respective number of coded amino acids (aa) are given, separated by a single long intervening intron sequence in case of PAR-1 to -3, whereas PAR-4 has a very short intron. The length of intron is given in kilobases (kb). Protease cleavage site (↓) located on exon 2 shown with the respective amino acids (R = arginine; S = serine; K = lysine; T = threonine; G = glycine). Activating serine proteases known for PAR-1 to -4 are listed.

PAR-4, which is located on band number 12 of the short arm (p) of chromosome 19 (19p12) (Kahn and others 1998). The cDNA, which reveals the amino acid sequence of the protein, has been cloned from the human, mouse, and rat for all four receptor subtypes, PAR-1 (Vu and others 1991), PAR-2 (Nystedt and others 1994), PAR-3 (Ishihara and others 1997), and PAR-4 (Xu and others 1998). PAR-1, which was the 1st receptor of this family to be cloned, is often referred to as the thrombin receptor and represents the prototype of this receptor family.

All four PAR subtypes share high homology between them and are conserved across species. Figure 2 describes the genomic organization of the human PAR genes with the protease cleavage site on exon 2 and the proteases known to activate PARs. The four subtypes show an identical gene structure, comprising two exons (coding regions), which are separated by a very long single intron (noncoding intervening region) of 4 to 22 kb in case of PAR-1 to -3. Again, PAR-4 is distinct because the intron is very short, only 0.274 kb (Kahn and others 1998). In all four cases, a short first exon encodes the 5′-untranslated sequence, the start codon and a signal peptide, whereas the second, large exon encodes the receptor protein and the 3′-untranslated sequence (Kahn and others 1998).

PAR activation by agonist binding results in a conformational change of the receptor, which leads to interaction of the receptor with G proteins, a heterotrimeric complex made up of three different subunits, designated α, β, and γ. The receptor activation causes a catalytic exchange of GTP for GDP on the α-subunit of the G protein. The α-subunit and $\beta\gamma$ heterodimer can each activate different effector enzymes or ion channels. This is terminated when GTP is hydrolyzed. Then the G proteins return to their inactive state. Among all PARs, for PAR-1 the signaling mechanism has been mostly studied

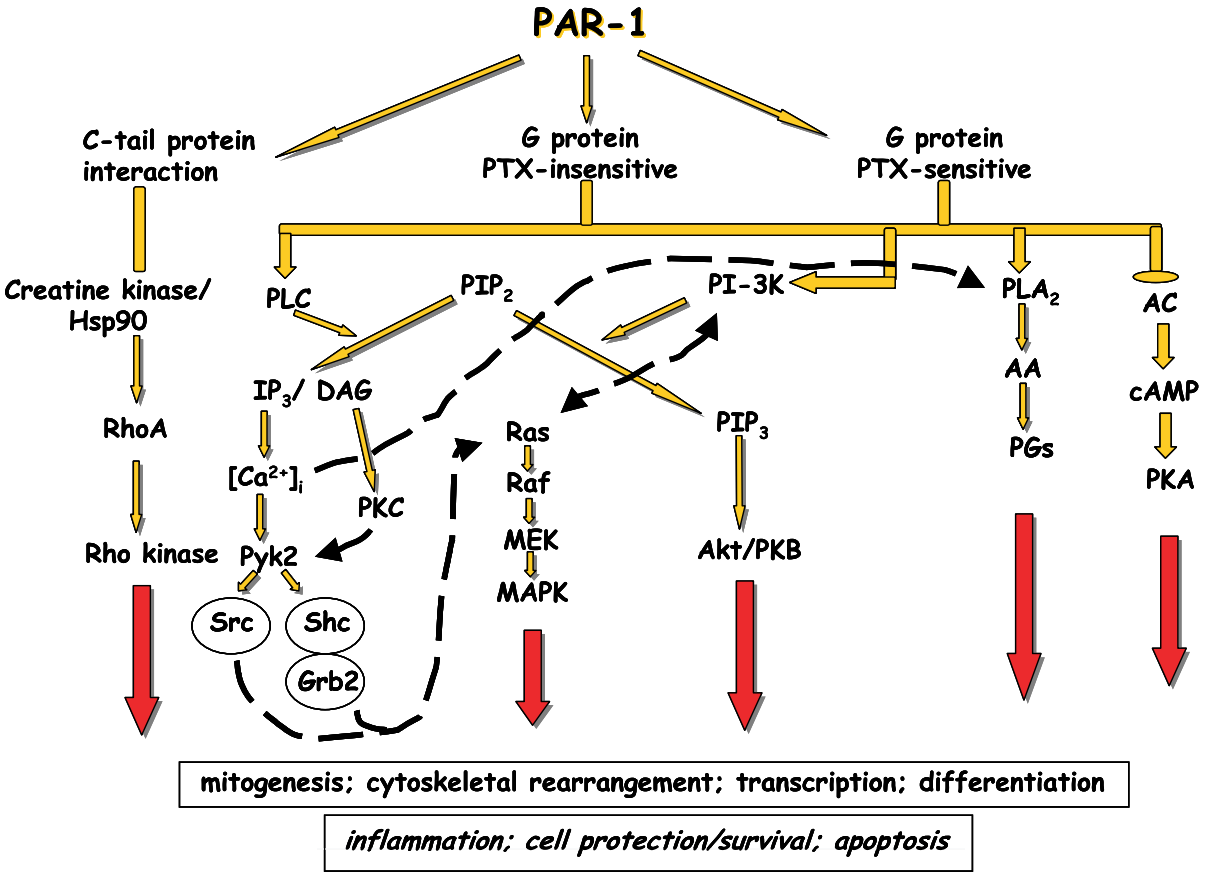

Fig. 3. Signaling pathways regulated through activation of protease-activated receptor (PAR)-1: Signaling pathways downstream of PAR-1 activation utilize heterotrimeric G proteins coupled to intracellular loop 3. Signal from G proteins (PTX-insensitive) results in activation of PLC, followed by generation of IP_3 and DAG and finally leading to phosphorylation of the MAPK p42/44. On the other hand, PTX-sensitive G proteins activate PI-3K pathway, which also results in phosphorylation of MAPK. PI-3K is also involved in the formation of PIP_3 from PIP_2 in the plasma membrane to activate then Akt/PKB. Activation of calcium-dependent cytosolic PLA_2 leads to release of AA, which is involved in the synthesis of PGs. PTX-sensitive G proteins control inhibition of cAMP pathway by inhibiting adenylyl cyclase. The signal produced from all these pathways ultimately results in effector functions, such as proliferation, transcription, cytoskeleton rearrangement, inflammation, apoptosis, and cell death or protection. The carboxy tail (C-tail) of PAR-1 can interact directly with proteins like creatine kinase and Hsp90, leading to activation and translocation of RhoA to plasma membrane, followed by activation of Rho kinase and subsequent rearrangement of cytoskeleton. Abbreviations: PTX = pertussis toxin; Hsp90 = heat shock protein 90; PI-3K = phosphatidylinositol 3-kinase; PIP_2 = phosphatidylinositol 4,5-bisphosphate; PIP_3 = phosphatidylinositol 3,4,5-triphosphate; PKB = protein kinase B/Akt; Src = Src tyrosine kinase; Shc = adaptor protein in the small GTPase pathway; Grb2 = growth factor receptor-bound protein; Ras and Raf = small GTPases; MAPK = mitogen-activated protein kinase; PLC = phospholipase C; IP_3 = inositol 1,4,5-trisphosphate; DAG = diacylglycerol; Pyk2 = proline-rich tyrosine kinase-2; PKC = protein kinase C; PLA_2 = phospholipase A_2; AA = arachidonic acid; PGs = prostaglandins; AC = adenylyl cyclase; cAMP = cyclic adenosine monophosphate; PKA = protein kinase A.

and is therefore best understood. Figure 3 highlights some of the intracellular signaling pathways initiated by PAR-1 activation. Agonist activation of PAR-1 in some cases results in inhibition of adenylyl cyclase, the enzyme that synthesizes the second messenger cAMP through interaction with inhibitory G proteins. Most frequently, there is stimulation of phospholipase C (PLC) via G_q protein. Activated PLC, which is a calcium-dependent enzyme, uses plasma membrane phosphoinositide, phosphatidyl inositol 4,5-bisphosphate (PIP_2) as a substrate and hydrolyzes it into inositol (1,4,5) trisphosphate (IP_3) and diacylglycerol (DAG). Furthermore, generation of IP_3 leads to mobilization of intracellular Ca^{2+}, whereas DAG participates in the activation of protein kinase C (PKC), an enzyme involved in phosphorylation of several kinds of amino acids. Another family of enzymes involved in PAR-1 signaling are mitogen-activated protein kinases (MAPK). MAPK play a central role in growth, proliferation, development, and survival of all eukaryotic organisms and are involved in phosphorylation of transcription factors, cytoskeletal proteins, and other protein kinases. In mammalian cells, three major MAPK pathways have been characterized; one of them is called p42/44 or extracellular signal-regulated kinase.

The PAR signaling mechanism in astrocytes is an example of an extensively studied pathway (Wang, Ubl, Stricker, and others 2002). We have furnished proof that stimulation by thrombin or PAR-1–activating peptide in cultured rat astrocytes leads to proliferation, which is

mediated via the MAPK pathway, as also included in Figure 3 (Wang, Ubl, Stricker, and others 2002). Astrocytes are a major type of glial cells in the brain and play an active role in brain function by influencing, and possibly even directing, the activity of neurons. Astrocytes undergo proliferation as a result of tissue injury. Generation of the other types of mature brain cells including neurons (neurogenesis) in the adult brain, is an integral feature of astrocytes. It is therefore important to understand the mechanism underlying the proliferation of astrocytes in the CNS. The stimulus by thrombin or PAR-1–activating peptide is induced through activation of PAR-1 and proceeds via two parallel intracellular pathways. One pathway involves activation of pertussis toxin (PTX)-sensitive G_i protein/(βγ-subunits). PTX is not only responsible for causing whooping cough disease, but it also elicits a variety of physiological and cellular effects including regulation of adenylyl cyclase. From G_i protein, the signal travels to phosphatidylinositol 3-kinase and ultimately results in MAPK phosphorylation (Fig. 3). The second pathway after PAR-1 activation connects G_q protein-PLC activation resulting in IP_3 and DAG generation with subsequent rise in intracellular calcium level and activation of the PKC pathway (Wang, Ubl, Stricker, and others 2002). Furthermore, we could also show that phosphorylation of proline-rich tyrosine kinase-2 (Pyk2) occurs via thrombin-mediated PAR-1 activation in cultured rat astrocytes (Wang and Reiser 2003). Pyk2, which is also known as calcium-dependent tyrosine kinase, is a nonreceptor tyrosine kinase. Pyk2 has been proved to link GPCRs to MAPK activation by recruiting several mediator proteins, such as Src, Shc, and Grb2, which are depicted in Figure 3 (Lev and others 1995). Pyk2 is highly sensitive to increased intracellular calcium levels and can be readily activated by signals that raise the Ca^{2+} concentration in the cell (Lev and others 1995). Another recent study from our laboratory, using an oligodendrocyte cell line, has revealed that intracellular calcium increase induced by agonist-stimulated PAR-1 activation mainly results from Ca^{2+} release from intracellular stores. Studies performed to elucidate the calcium signaling mechanism verify that a PAR-1–mediated rise in calcium occurs via PTX-insensitive G protein. Activation of PLC and liberation of IP_3 are events preceding Ca^{2+} release from the stores (Wang and others 2004). We have also shown that thrombin-stimulated release of the polyunsaturated fatty acid AA by astrocytes is mediated via the calcium-dependent phospholipase A_2 (PLA_2) (Strokin and others 2003).

Compared with the large number of reports exploring the PAR-1 signaling pathway, little is known about the mechanism of PAR-2 intracellular signaling. Trypsin and PAR-2–activating peptide can stimulate IP_3 formation and Ca^{2+} rise in numerous cell types via heterotrimeric G proteins (Schultheiss and others 1997). PAR-2 signaling via a PTX-sensitive pathway has been found to be associated with a tyrosine phosphatase, called SHP-2 (Yu and others 1997), which enhances the agonist-stimulated PAR-2 mitogenic pathway.

Another important aspect of PAR signaling is receptor desensitization and resensitization. Desensitization involves loss of functional response after seconds or minutes of agonist stimulation, due to the uncoupling of the activated receptor from the G proteins as a result of receptor phosphorylation. On the other hand, resensitization means recovery of cellular response to proteases, which requires intact receptors at the cell surface. A study undertaken to investigate the mechanisms of PAR-2 desensitization in transfected kidney epithelial cells has made clear that PAR-2–mediated calcium mobilization can be desensitized by irreversible receptor cleavage, followed by PKC-mediated termination of signaling, and ultimately by targeting the internalized cleaved receptor on lysosomes for degradation. Resensitization of PAR-2 occurs via mobilization of intracellular pools of receptor from Golgi stores and synthesis of new receptors (Böhm, Khitin, and others 1996). Furthermore, the same group has shown that β-arrestin, an adaptor protein, mediates agonist-induced endocytosis of PAR-2 (Dery and others 1999). Use of several mutants truncated at the cytoplasmic tail of PAR-2 in a recent study has highlighted the fact that different regions within the C-terminus of PAR-2 are involved in different downstream signaling pathways, and different PARs utilize distinct domains for the same downstream effector function (Seatter and others 2004). Work done in our laboratory was able to demonstrate that thrombin induces a dose-dependent desensitization of PAR-1 in rat primary astrocytes and resensitization occurs via synthesis of new receptors (Ubl and others 2000). Phosphorylation of the activated PAR-1 cytoplasmic tail results in receptor desensitization and internalization. However, the desensitization and internalization pathways are mediated by different adaptor proteins. Although PAR-1 desensitization is regulated by β-arrestin, internalization of PAR-1 is through a dynamin- and clathrin-dependent pathway (Paing and others 2002). Using chimeras, it was found that the cytoplasmic tail of PAR-1 determines the trafficking of internalized receptor to lysosomes (Trejo and Coughlin 1999).

To gain further insight into the PAR physiology and its involvement in signaling, it is important to functionally characterize the parts played by different regions of the receptor. Not much is known yet with regard to the function of the C-tail of the receptor and its role in the intracellular signaling pathway. Therefore, to identify the proteins directly interacting with the C-tail of PAR-1, two studies have been done (Mahajan and others 2000; Pai and others 2001). It was discovered that a cytosolic brain isoform of creatine kinase interacts with the C-tail of the rat PAR-1 and this interaction was found to be involved in cell shape change of neurons (Mahajan and others 2000), which is included in Figure 3. Creatine kinase is an ATP-generating enzyme that regulates ATP homeostasis within subcellular compartments. Cytosolic creatine kinase provides energy in the form of ATP, required for PAR-1 signal transduction leading to actin reorganization (Mahajan and others 2000). Actin, a com-

ponent of the cytoskeleton, can undergo rearrangement to produce cell movement. The PAR-1 signaling pathway involves activation and translocation of RhoA, a monomeric GTP-binding protein to the plasma membrane where it subsequently activates Rho kinase that relays signals to the cytoskeleton, finally leading to actin rearrangement. The study showed that PAR-1 signaling and PAR-1-mediated cellular shape changes are inhibited when either creatine kinase levels or its ATP-generating capacity are reduced. However, the PAR-1–creatine kinase complex has no role to play in thrombin-stimulated intracellular calcium release by astrocytes.

Another study by the same group revealed that the heat shock protein Hsp90 is an interaction protein of the C-tail of human PAR-1 (Pai and others 2001). Hsp90 is a molecular chaperone with functions under both stress and nonstress conditions. Molecular chaperones are proteins aiding in the correct folding of proteins and thereby preventing formation of an inactive conformation. The change in astrocyte morphology and hence cytoskeletal rearrangement was revealed to be mediated via the PAR-1-Hsp90 complex (Pai and others 2001). Similar to creatine kinase, Hsp90 is not involved in thrombin-mediated calcium release either (Pai and others 2001). Recently we have identified a PAR-2–interacting protein with resemblance to a family of small heat shock proteins in the retina, which is part of the CNS (Rohatgi and others, unpublished data). This protein, α crystallin A, which is a 20 kDa protein, co-localizes both with neurons and astrocytes and shows a cytosolic distribution as seen in our analysis. It was initially described as a lens-specific protein required to maintain lens transparency, but in the last few years it has been demonstrated that α crystallin A occurs in several other tissues including brain, heart, spleen, and retina. Now α crystallin A is known to co-localize with actin cytoskeleton and to possess antiapoptotic activity. These functions suggest a possible role of α crystallin A either in PAR-2–mediated cell shape change or cell survival in brain cells.

Concluding Remarks

The varied functions of thrombin and PARs both in normal and pathological states of the brain summarized in this review emphasize the need to further investigate the functioning of proteases and their receptors in the nervous system. These studies will help to analyze the areas where modulating PAR activity can prove useful. They provide two promising potential therapeutic approaches for treating neurodegenerative diseases: first, downstream effector molecules involved in thrombin and PAR-mediated signaling pathways in the brain and, second, agents that inhibit PAR activation during diseased states.

Appendix

Abbreviations

AA = Arachidonic acid
CNS = Central nervous system
DAG = Diacylglycerol
GPCR = G protein-coupled receptor
HIV = Human immunodeficiency virus
IP_3 = Inositol 1,4,5-trisphosphate
MAPK = Mitogen-activated protein kinase
NMDA = *N*-methyl D-aspartate
OGD = Oxygen glucose deprivation
PARs = Protease-activated receptors
PI-3K = Phosphatidylinositol 3-kinase
PIP_2 = Phosphatidyl inositol 4,5-bisphosphate
PKC = Protein kinase C
PLA_2 = Phospholipase A_2
PLC = Phospholipase C
PN-1 = Protease nexin-1
PNS = Peripheral nervous system
PTX = Pertussis toxin
Pyk2 = Proline-rich tyrosine kinase-2
RT-PCR = Reverse transcription-polymerase chain reaction
SN = Substantia nigra
TNF-R = Tumor necrosis factor-receptor

Glossary

CD40: A receptor molecule on the cell surface of all mature B lymphocytes, most B-cell malignancies, and monocytes, dendritic cells (in the nervous system), endothelial cells (within blood vessels), and epithelial cells. CD40 is a member of the tumor necrosis factor receptor superfamily. Together with CD40 ligand, it is an important contributor to inflammatory processes.

Endothelin: Endothelins are a family of potent vasoconstrictor peptides elevating blood pressure, comprising three isoforms. Endothelin is found in a host of mammalian species and has a potent effect in both cardiovascular and central nervous systems. The endothelin receptor is a seven-transmembrane domain receptor coupled to G proteins. Two structurally and functionally distinct receptors for endothelin, ETA and ETB, are known. Both ETA and the ETB receptors play varying roles in mediation of vasopressor actions.

GPCR: G protein-coupled receptors that mediate their actions by stimulating guanine nucleotide binding regulatory proteins (G proteins). They share structural as well as functional similarities. The structural motif characteristic of receptors of this class includes seven hydrophobic transmembrane domains linked by hydrophilic loops. The G proteins bind the GDP and GTP. They are heterotrimers, that is, made of three different subunits, associated with the inner surface of the plasma membrane and transmembrane receptors.

Ischemia: Ischemia is a condition in which the oxygen-rich blood flow to a part of the body is restricted. An example of experimental in vitro ischemia is oxygen-glucose deprivation (OGD). In this model, organotypic cultures (hippocampal or corticostriatal slice cultures) are subjected to OGD to investigate mechanisms concerning ischemic cell death and neuroprotection.

Molecular chaperone: Proteins associated with a second protein during part of its folding process. However, once folding is complete (or even before) the chaperone leaves its current protein molecule and goes on to support the folding of another one.

NMDA receptor: The *N*-methyl-D-aspartate receptor is a receptor for the neurotransmitter glutamate, which is the most important excitatory neurotransmitter in the brain. NMDA receptor is both a receptor and an ion channel, thus a ligand-gated ionic channel. *N*-methyl-D-aspartate is the specific agonist of the NMDA receptor. The receptor is involved in the toxic effects of excessive glutamate and in many other processes such as synaptic plasticity and target recognition.

Pyk2: Proline-rich tyrosine kinase 2 is a 116 kDa non-receptor tyrosine kinase that belongs to the focal adhesion kinase family of tyrosine kinases. Pyk2 is a ubiquitously expressed protein, but most abundant in the brain. Pyk2 is localized at cell-cell contacts and is very sensitive to stimuli that increase either intracellular calcium levels or activate protein kinase C. Pyk2 links G proteins and MAPK signaling pathways.

RhoA: RhoA is a member of the Ras homology family of small GTPases. These proteins cycle from their active GTP-bound to their inactive GDP-bound state by hydrolyzing GTP to GDP. Several regulatory proteins can influence RhoA by increasing or decreasing its GTPase activity. RhoA is involved in regulation of cytoskeleton and cellular response to stress.

TNF-R: Tumor Necrosis Factor Receptor is a family of related proteins, involved in cytokine-mediated apoptosis. These are surface-expressed transmembrane proteins that require clustering of the receptors by trimeric ligands. Several members of the TNF-R family are characterized by a homologous cytoplasmic domain referred to as a Death Domain. The tumor necrosis factor-α is a proinflammatory cytokine. The main sources of TNF-α are stimulated fibroblasts, macrophages, T cells, B lymphocytes, mast cells, and glial cells. TNF-α can also be detected in the cerebrospinal fluid.

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