

Perspectives in Pharmacology

Protease-Activated Receptors: New Concepts in Regulation of G protein-Coupled Receptor Signaling and Trafficking

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Abbreviations: AP-2, adaptor protein complex-2; β_2 AR, β_2 -adrenergic receptor; β arr, β -arrestin; GASP, GPCR-associated sorting protein; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRKs, G protein-coupled receptor kinases; MAPK, mitogen-activated protein kinase; MEFs, mouse embryonic fibroblasts; PARs, protease-activated receptors; PLC, phospholipase C; SNX, sorting nexin; SPR, substance P receptor

ABSTRACT

Most G protein-coupled receptors (GPCRs) are reversibly activated upon ligand binding. However, activation of protease-activated G protein-coupled receptors (PARs) occurs through an irreversible proteolytic event that results in the generation of a tethered ligand that cannot diffuse away. This unusual mode of PAR activation raises important questions regarding the mechanisms responsible for termination of receptor signaling. There are currently four members of the PAR family. Thrombin activates PAR1, PAR3 and PAR4, whereas multiple trypsin-like serine proteases activate PAR2. The regulation of signaling by PAR1 has been extensively studied, whereas considerably less is known about the other PARs. It has been demonstrated that rapid termination of PAR1 signaling is critical in determining the magnitude and kinetics of the cellular protease response. Therefore, elucidating the molecular mechanisms involved in the regulation of PAR signaling is essential to fully understand protease-mediated responses. Recent findings indicate that novel mechanisms contribute to PAR1 desensitization, internalization and down-regulation. This review focuses on the intracellular mechanisms that regulate PAR signaling and the recent progress in developing inhibitors of PAR signaling.

Family of PARs

The family of PARs currently includes four members: PAR1, PAR2, PAR3 and PAR4. The coagulant protease thrombin is the physiological activator of PAR1, PAR3 and PAR4; however, other proteases can cleave these receptors and may contribute to their function *in vivo* (Coughlin 2000; O'Brien et al. 2001). PAR2 is activated by multiple trypsin-like serine proteases including trypsin, tryptase, and coagulation proteases upstream of thrombin, factors VIIa and Xa, but not by thrombin (Coughlin and Camerer 2003). Thrombin, and perhaps other coagulant proteases, mediates responses critical for hemostasis and thrombosis, as well as inflammatory and proliferative responses triggered by tissue damage (Coughlin 2000). These responses are elicited predominantly by PARs expressed in a variety of cell types associated with the vascular system. Thrombin-induced signaling responses in the brain also appear to be mediated by PARs (Xi et al. 2003). PARs are found on sensory nerves and contribute to neurogenic edema and nociception. Interestingly, PAR expression has also been detected in dendritic cells and T cells; however, the functional significance of PARs in these cell types is not known. PARs play important roles in normal physiology and disease, however, in many cases the particular protease and PAR that function in specific cellular process remains to be defined.

PAR Activation and G protein Signaling

The mechanism of PAR activation was initially established for PAR1 (Vu et al. 1991), and appears to be a general paradigm for other PARs. PARs are activated by an unusual irreversible proteolytic mechanism in which the protease binds to and cleaves the amino-terminal exodomain of the receptor. This cleavage generates a new amino terminus that functions as a tethered ligand by binding intramolecularly to the body of the receptor to initiate transmembrane signaling. Synthetic peptides, which represent the newly formed amino terminus, activate the receptor independent of protease and receptor cleavage. The activation of PAR3, however, may vary from this model. Human PAR3 is activated by thrombin when ectopically expressed in COS-7 cells, but peptide agonists that mimic the newly exposed amino terminus fail to elicit signaling responses (Ishihara et al. 1997). This suggests that perhaps cleavage alone is sufficient to activate PAR3. Interestingly, murine PAR3 does not appear to signal by itself and instead serves a cofactor for cleavage and activation of PAR4 (Nakanishi-Matsui et al. 2000). Thrombin binds to the amino-terminal exodomain of PAR1 and PAR3 by interacting with sequences

amino- and carboxyl-terminal to the thrombin cleavage site. The carboxyl terminal sequence confers high affinity binding to thrombin and is not present in the PAR4 exodomain. Thus, PAR4 requires higher thrombin concentrations for activation compared to the other receptors (Kahn et al. 1999). Regardless, PARs are irreversibly activated, and thus the mechanisms that contribute to termination of signaling are critical determinants of the magnitude and kinetics of the protease response in cells.

PARs are seven transmembrane G protein-coupled receptors and most likely elicit signaling responses according to the classic paradigm established for other GPCRs. That is, upon ligand activation of PARs, conformational changes in the receptor promotes interaction with heterotrimeric G proteins. In common with several other GPCRs, PAR1 interacts with multiple G protein subtypes (reviewed in MacFarlane et al. 2001). Several early studies indicated that PAR1 couples to inhibition of cAMP accumulation through G_i and stimulates phospholipase C (PLC)-catalyzed hydrolysis of phosphoinositides to stimulate production of inositol trisphosphate and diacylglycerol through G_q . More recent studies have illustrated coupling of PAR1 to $G_{12/13}$ (Offermanns et al. 1994), however, whether activation of PAR1 modulates $G_{12/13}$ effectors such as Rho guanine nucleotide exchange factors (GEFs) and PLC- ϵ remains to be determined (Lopez et al. 2001; Mao et al. 1998). The extent to which PAR1 couples to each of these pathways in a particular cell type presumably depends upon the G protein and effector repertoire expressed in the cell. Moreover, the precise mechanism(s) by which PAR1 desensitizes to these distinct G protein subtypes remains to be determined. In contrast to PAR1, studies that directly assess the coupling of other PARs to distinct G protein subtypes have not been reported. However, activation of PAR2, human PAR3 and PAR4 cause increases in inositol phosphates and mobilization of Ca^{2+} in a variety of cell types (Bohm et al. 1996; Ishihara et al. 1997; Shapiro et al. 2000), suggesting that these receptors are capable of activating G_q and/or G_i signaling responses.

GRK-mediated PAR Signal Regulation

GPCRs are initially desensitized by rapid phosphorylation of activated receptors by G protein-coupled receptor kinases (GRKs) and other kinases (Krupnick and Benovic 1998). In many cases phosphorylation enhances receptor affinity for arrestin, and arrestin binding prevents receptor-G protein interaction, thereby uncoupling the receptor from signaling. It is possible that

proteolytic cleavage of PARs could result in sustained activation of the receptor by the tethered ligand that does not diffuse away. However, signaling by PAR1 is rapidly terminated despite the irreversible proteolytic mechanism of receptor activation. It was previously demonstrated that the cumulative phosphoinositide hydrolysis response to thrombin precisely correlates with the absolute rate of receptor cleavage during a given time (Ishii et al. 1993). This suggests that each activated PAR1 signals, generates a defined amount of second messenger, and then shuts off (at least in terms of G_q activation).

The rapid termination of PAR1 signaling appears to be regulated at least in part by GRK-mediated phosphorylation. Overexpression of GRK3 and GRK5 enhances PAR1 phosphorylation and markedly inhibits inositol phosphate accumulation (Ishii et al. 1994; Tirupathi et al. 2000). A PAR1 mutant in which all of the serines and threonines in the cytoplasmic tail (C-tail) are converted to alanines signals greater than wild-type receptor and is neither extensively phosphorylated nor inhibited by GRK3 overexpression (Ishii et al. 1994; Nanevycz et al. 1996). This suggests that the C-tail is the major site for GRK-mediated termination of PAR1 signaling (Fig.1). Interestingly, however, the PAR1 mutant that lacked all potential C-tail phosphorylation sites retained the ability to confer thrombin-dependent responses in a *Xenopus laevis* oocyte system and COS-7 cells (Ishii et al. 1994; Nanevycz et al. 1996), suggesting that additional mechanism(s) beyond GRK phosphorylation exist for termination of PAR1 signaling (Fig.2). Thus, it is likely that other mechanisms besides C-tail phosphorylation contribute to rapid termination of PAR1 signaling in cells. For example, β arrestin binding to activated PAR1 independent of phosphorylation maybe sufficient to induce desensitization. Alternatively, phosphorylation at other sites in the receptor and/or second messenger kinases could also play a critical role in regulating PAR1 signaling. Indeed, the second messenger protein kinase C is capable of modulating PAR1 signaling. Direct activation of protein kinase C with phorbol esters leads to phosphorylation of PAR1 and heterologous desensitization of receptor signaling in endothelial cells (Ishii et al. 1994; Yan et al. 1998).

The C-tail of PAR2 contains multiple potential sites of phosphorylation (Fig.1), but studies that assess the function of GRKs in termination of PAR2 signaling have not been reported. However, pharmacological inhibitors of PKC have been used to suggest a function for PKC in PAR2 desensitization (Bohm et al. 1996). PAR4 does not appear to undergo agonist-promoted phosphorylation when overexpressed in Rat1 fibroblasts (Shapiro et al. 2000),

despite the presence of several potential sites of phosphorylation (Fig.1). Moreover, the function of GRKs in the termination of PAR4 signaling in fibroblasts or other cell types is not known. The cytoplasmic tail of PAR3 is considerably shorter than the C-tail of other PARs (Fig.1) and the regulatory mechanisms responsible for termination of PAR3 signaling have not been determined.

β arrestins and PAR Signaling

The nonvisual arrestins, arrestin2 and arrestin3 (also termed β arrestin1 and β arrestin2) are ubiquitously expressed and play critical roles in regulating the signaling of most GPCRs (Kohout and Lefkowitz 2003), suggesting that arrestins function in termination of PAR signaling. We recently assessed the function of β arrestins in PAR1 signaling and trafficking using mouse embryonic fibroblasts (MEFs) derived from β arrestin knockouts (Paing et al. 2002). MEFs provide a mammalian cell system in which β arrestin function can be studied without relying on ectopic overexpression of wild-type or dominant-negative versions of the protein. In wild-type cells expressing both isoforms of β arrestin and endogenous PAR1, thrombin signaling rapidly desensitized (Paing et al. 2002). In contrast, the rate of PAR1 desensitization was markedly impaired in cells lacking both β arrestin isoforms. In cells expressing comparable levels of surface FLAG-tagged PAR1, signaling to phosphoinositide hydrolysis was similarly enhanced in β arr1,2 deficient cells compared with wild-type control cells (Paing et al. 2002). The differences in PAR1 signaling observed in wild-type and β arr1,2 deficient cells are not due to altered expression of G proteins and/or effector PLC enzymes, since we have determined that similar amounts of these signaling molecules are present in both cell types (unpublished observations). These findings are the first to demonstrate a function for β arrestins in the regulation of PAR1 coupling to G protein signaling. Both phosphorylation and β arrestin binding contribute to PAR1 desensitization (Fig. 2). However, the relative contribution of these pathways to the termination of PAR1 signaling remains to be determined. The function of β arrestins in the regulation of G protein signaling by other PARs is not known.

The functional differences of β arr1 *versus* β arr2 in the regulation of GPCR signaling are poorly understood. MEFs derived from β arrestin single knockouts (lacking only β arr1 or β arr2 expression) present a unique opportunity to define the roles of the individual β arrestins in the

regulation of PAR signaling. The β arrestin isoforms are expressed at relatively similar levels (Kohout et al. 2001), thus valid comparisons can be made between the cell lines. We recently observed that the rate of PAR1 desensitization was markedly impaired in β arr1 lacking cells compared to β arr2 deficient cells and wild-type controls (Paing et al. 2002). The predominant effect of β arr1 *versus* β arr2 in the regulation of PAR1 uncoupling to G_q signaling has also been observed in COS-7 cells (C.-H. Chen, M. M. Paing and J. Trejo, manuscript in preparation). Together these studies suggest the possibility that β arr1 is the critical mediator of PAR1 desensitization at least to G_q signaling. Moreover, this is the first example of the isoforms of β arrestins differentially regulating GPCR desensitization. PAR1 couples to G_q , G_i and $G_{12/13}$ and it remains to be determined whether β arrestins exhibit specialized function(s) in regulating PAR1 uncoupling from specific G protein subtypes. The possibility that β arrestin isoforms differentially regulate PAR signaling is tantalizing.

Internalization of PARs

Internalization of GPCRs contributes to signal termination by removing activated receptors from G proteins and signaling effectors. Given the irreversible nature of PAR activation, internalization and lysosomal sorting of proteolytically activated PARs may be particularly important for termination of receptor signaling. In many cases, arrestins facilitate GPCR internalization by binding to clathrin and β 2-adaptin of the adaptor protein complex-2 (AP-2) complex, thereby linking GPCRs to the endocytic machinery (Ferguson 2001). The vast majority of GPCRs internalize from the plasma membrane via a clathrin- and dynamin-dependent pathway. Although some GPCRs internalize via a distinct non-dynamin dependent pathway (Pals-Rylaarsdam et al. 1997; Vickery and von Zastrow 1999). Dynamin is a GTPase that regulates budding of clathrin-coated pits and in some cells facilitates detachment of caveolae (Schmid et al. 1998). Immunoelectron microscopy first was used to demonstrate clustering of activated PAR1 in clathrin-like coated pits in human megakaryoblastic cells (Hoxie et al. 1993). We subsequently reported that PAR1 used a clathrin- and dynamin-dependent pathway for internalization based on observations that activated PAR1 was rapidly recruited to clathrin-coated pits and internalization was blocked by dominant-negative dynamin and clathrin hub mutants in HeLa cells (Trejo et al. 2000). These observations, together with the

phosphorylation-dependence of PAR1 internalization (Shapiro et al. 1996), raise the possibility that arrestins function in PAR1 internalization.

Interestingly, agonist-triggered PAR1 internalization occurs normally in MEFs that lack β arrestin expression (Paing et al. 2002), suggesting that β arrestins are not required for internalization of activated PAR1. Degradation of activated PAR1 was also observed in β arrestin deficient cells. In contrast, β_2 AR receptor fails to internalize in the same β arrestin lacking cells that robustly internalize PAR1 (Paing et al. 2002), consistent with a role for β arrestins in β_2 AR internalization as previously reported (Kohout et al. 2001). Although arrestins are not essential for internalization, phosphorylation of activated PAR1 was shown to be required for internalization in MEFs (Paing et al. 2002). Arrestin-independent PAR1 internalization also occurred via a clathrin- and dynamin-dependent pathway in these cells. Studies using COS-7 cells are consistent with a β arrestin-independent pathway for PAR1 internalization. In COS-7 cells which express low levels of β arrestins, activated β_2 AR is modestly internalized, and co-expression of β arrestins significantly enhance agonist-induced receptor internalization (Zhang et al. 1996). In contrast, in COS-7 cells transiently expressing PAR1, agonist stimulates robust PAR1 internalization (Shapiro et al. 1996), and co-expression of either β arr1 or β arr2 fails to enhance internalization of activated PAR1 (unpublished observations). Although the effect of dominant-negative β arrestins on PAR1 internalization has not been determined, the available data indicate that activated PAR1 is internalized in an arrestin-independent pathway, whereas regulation of receptor G protein coupling is clearly arrestin-dependent. This distinct regulation of PAR1 signaling and trafficking by arrestin is consistent with a previous study in which mutation of specific C-tail phosphorylation sites impaired termination of signaling but failed to alter PAR1 internalization (Hammes et al. 1999). Interestingly, phosphorylation of a C-terminal serine residue (Ser-412) in β arr1 regulates its endocytic but not desensitization function (Lin et al. 1997). Thus, it is possible that, upon PAR1 activation, β arr1 is recruited to the receptor and fails to undergo dephosphorylation and, therefore, is unable to promote receptor interaction with clathrin. This possibility remains to be tested; whether β arr2 is regulated similarly is not known.

The mechanism by which activated PAR1 is recruited to clathrin-coated pits is not known. It appears as though PAR1 internalization through clathrin-coated pits is controlled by multiple regulatory mechanisms (Fig.3). The first involves phosphorylation of the C-tail that

occurs at several alternative sites (Hammes et al. 1999), any of which is sufficient for internalization. The second involves interaction with an adaptor protein (other than β arrestins) that presumably binds to a sorting sequence present in a cytoplasmic domain of PAR1 and recruits the receptor to clathrin-coated pits. Our previous studies have shown that the C-tail of PAR1 is essential for internalization and lysosomal degradation (Shapiro et al. 1996; Trejo and Coughlin 1999), and presumably contains important information for this process. Several specific sorting signals used for clathrin-dependent endocytosis have been identified in the cytoplasmic tails of transmembrane proteins including di-leucine and tyrosine-based motifs (Kirchhausen 1999). PAR1 contains a di-leucine motif near its C-terminus but mutation of this sequence did not cause significant defects in receptor trafficking (unpublished observations). A highly conserved tyrosine-based sorting motif, YSIL, is also present in the C-tail of PAR1 but is absent in the C-tail of other PARs. Interestingly, a PAR1 mutant in which Tyr-383 and Leu-386 are converted to alanine (Y383A, L386A) displays a significant loss of agonist-induced internalization and degradation when transiently expressed in HeLa cells, whereas signaling by this mutant PAR1 is unperturbed (M. M. Paing and J. Trejo, manuscript in preparation). This YSIL motif is strikingly similar to other receptor sorting sequences known to directly bind the μ 2 subunit of the AP-2 complex (Kirchhausen 1999). Thus, a role for AP-2 function in PAR1 internalization is possible.

The molecular mechanisms that mediate internalization and lysosomal sorting of other PARs are not clearly understood. Activation of PAR2 causes rapid and transient redistribution of β arr1 fused to green fluorescent protein (GFP) when overexpressed in KNRK cells (Dery et al. 1999). Although overexpression of wild-type β arr1 did not enhance PAR2 internalization, a C-terminal fragment of β arr1³¹⁹⁻⁴¹⁸ partially inhibited receptor internalization. Because mutant β arr1³¹⁹⁻⁴¹⁸ does not directly interact with the receptor but instead binds constitutively to clathrin it has the potential to non-selectively inhibit cargo that utilizes clathrin-coated pits for internalization. Thus, these studies do not exclude the possibility that another adaptor protein besides β arrestin recruits PAR2 for internalization from the plasma membrane. Interestingly, activated PAR4 is internalized at a much slower rate than PAR1 in Rat1 fibroblasts (Shapiro et al. 2000), suggesting that distinct mechanisms regulate internalization of these receptors. It remains to be determined whether internalization of PAR2, PAR3 and PAR4 will proceed

through pathways similar to PAR1 or whether distinct pathways will regulate trafficking of these receptors.

Down-regulation of PARs

The process of down-regulation, a decrease in total receptor number, occurs after prolonged agonist exposure for most classic GPCRs. The regulation of GPCR protein levels occurs partially at the level of transcription and RNA stability. Trafficking of internalized GPCRs from endosomes to lysosomes is also critical for down-regulation (Tsao et al. 2001). Most classic GPCRs internalize, rapidly recycle, and slowly down-regulate. However, activated PAR1 and PAR2 internalize, sort predominantly to lysosomes and are rapidly degraded. Several studies suggest that PAR1 down-regulation by receptor internalization and lysosomal sorting, are required to terminate signaling by irreversibly activated receptors that remain at or return to the cell surface. Activation of human PAR1 ectopically expressed in Sf9 insect cells caused persistent signaling even after removal of ligand (Chen et al. 1996). These findings suggest that the tethered ligand of PAR1 is capable of signaling continuously in the absence of appropriate termination machinery, which Sf9 cells apparently lack. Consistent with this idea, a PAR1 chimera containing the C-tail of the substance P receptor (SPR), a classic GPCR, internalizes and recycles back to the cell surface and showed enhanced and prolonged signaling after activation with thrombin (Trejo et al. 1998). This prolonged signaling was apparently due to recycling and continued signaling by the receptors that return to the cell surface with their tethered ligands intact. Moreover, we recently demonstrated that trafficking of endogenous PAR1 is altered in metastatic breast carcinoma cells, such that activated receptor is not sorted to lysosomes and degraded (Booden et al. 2003). Consequently, activated PAR1 caused sustained signaling to phosphoinositide hydrolysis and mitogen-activated protein kinase (MAPK) even after thrombin withdrawal. Thus, internalization and lysosomal sorting of activated PAR1 is critical for the temporal fidelity of thrombin signaling.

The mechanisms involved in down-regulation of GPCRs by internalization and lysosomal sorting remains poorly understood. It is likely that cytosolic sequences of GPCRs, particularly in the C-tail, are important for direct interaction with molecules on transport vesicles. Interestingly, a function for ubiquitination of the β_2 AR and chemokine receptor CXCR4 in lysosomal sorting and receptor degradation in mammalian cells has recently been demonstrated (Marchese and

Benovic 2001; Shenoy et al. 2001). By contrast, ubiquitination of the δ -opioid receptor is not essential for internalization and postendocytic sorting to lysosomes (Tanowitz and Von Zastrow 2002). The ubiquitination of β arrestins also appears to be important for the ability of arrestin to facilitate internalization of the β_2 AR (Shenoy et al. 2001). Thus, direct ubiquitination of GPCRs and/or components of the endocytic machinery serve multiple functions in GPCR trafficking. Whether ubiquitination is involved in internalization and lysosomal sorting of activated PARs is not known.

Other recent studies have described interacting proteins that target GPCRs for lysosomal degradation. A protein termed GASP (GPCR-associated sorting protein) appears to be important for lysosomal degradation of the δ -opioid receptor and perhaps other GPCRs (Whistler et al. 2002). The binding of GASP to the C-tail of δ -opioid receptor promotes lysosomal sorting of the receptor, whereas a dominant-negative version of GASP blocks sorting of the receptor to the degradative pathway. It is not known whether GASP functions in lysosomal sorting of PARs. However, we recently found that sorting nexin 1 (SNX1) interacts with activated PAR1 and regulates lysosomal sorting of the receptor (Wang et al. 2002). Sorting nexins belong to a diverse group of cellular trafficking proteins. SNX1 contains a phospholipid-binding motif termed the phox homology (PX) domain, and a carboxyl terminal coiled-coil domain (Haft et al. 1998; Zhong et al. 2002). Overexpression of SNX1 C-terminus blocks delivery of internalized PAR1 from endosomes to lysosomes (Wang et al. 2002). The SNX1 C-terminus dimerizes with full length SNX1 suggesting that the SNX1 C-terminal domain acts by sequestering endogenous SNX1, thereby disrupting its function. It is also possible that by disrupting SNX1 function, the SNX1 C-terminus alters SNX2 function since SNX1 and SNX2 associate to form heterodimers in cells (Haft et al. 1998). These findings raise the distinct possibility that SNX2 is also involved in lysosomal sorting of PAR1, and perhaps other PARs. The precise mechanism by which SNX1 interacts with PAR1 and the sorting machinery to direct receptor trafficking to lysosomes is not known.

Inhibitors of PAR signaling

The development of PAR specific antagonists has been slow due in part to the unusual intramolecular tethered ligand mechanism of activation. However, PAR activation can be inhibited by strategies that block extracellular domains of the receptor. Thrombostatins, modified

bradykinin-derived blocking peptides, appear to directly bind and inhibit PAR1 activation and do not act as thrombin inhibitors (Derian et al. 2003). Monoclonal antibodies generated against the cleavage site of PAR1 have also been used to block cleavage and activation of PAR1 (O'Brien et al. 2001). Small molecule PAR1 antagonists have also been generated based on the structure of the peptide ligand for PAR1 (MacFarlane et al. 2001). These antagonists function by blocking interaction of the newly exposed tethered ligand with binding sites on the extracellular face of the receptor but do not inhibit thrombin binding or receptor cleavage. However, because of structural similarities to activating peptides of other PARs many of these molecules lack PAR1 selectivity. Several recently developed peptide-mimetic and non-peptide PAR1 antagonists appear to be more selective (Derian et al. 2003; Hollenberg and Compton 2002). In addition to increased PAR1 selectivity, these compounds display relatively potent inhibitory actions against both thrombin and agonist-peptide stimulated responses. The development of effective PAR1 antagonists is in early stages, however, further characterization of the recently developed compounds will likely yield important information towards generating novel effective antagonists for PAR1 and other PARs.

In addition, other studies have described intracellular inhibitors that disrupt PAR-G protein interaction. The C-termini of G protein α subunits are critical for binding to their cognate GPCR and determining specificity. Peptides corresponding to the C-terminus of G_α subunits have been used to block PAR1 coupling to specific G protein subtypes in endothelial cells (Gilchrist et al. 2001). This strategy is useful to dissect out which G protein subtype mediates a particular response but lacks PAR specificity since these peptides would presumably block coupling of G proteins to all GPCRs expressed in the same cell. In another promising approach, cell permeable palmitoylated peptides corresponding to the third intracellular loop of PAR1 or PAR4 appear to be capable of inhibiting thrombin signaling (Covic et al. 2002). These lipid-modified peptides termed pepducins are thought to anchor in the plasma membrane and block interaction of the PAR intracellular loops with G proteins. However, cross-inhibitory effects of PAR4 pepducins on PAR1 activation occur and the precise mechanism by which pepducins elicit their inhibitory effects on thrombin signaling is not known. Thus, further development of these agents is necessary to determine whether they can be used to selectively inhibit PAR receptors.

In summary, PARs are irreversibly activated, thus the mechanisms that contribute to termination of signaling are critical determinants of the magnitude and kinetics of the protease

response in cells. The unusual irreversible proteolytic mechanism of PAR activation is clearly distinct from that involved in activation of other GPCRs. Thus, novel mechanisms appear to have evolved to deal with termination of signaling by these proteolytically activated GPCRs. The discussion above highlights some of the unusual aspects by which β arrestins function in the regulation of PAR1 signaling and trafficking. The internalization and lysosomal sorting of activated PAR1 is also critical for termination of receptor signaling. Clearly our understanding of these processes are based mostly on studies of PAR1 and it remains to be determined whether the other PARs will be similarly regulated.

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Footnotes

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Figure Legends

Fig.1. Cytoplasmic tail sequence alignment of PARs. The cytoplasmic tails of human PAR1, PAR2 and PAR3 are shown. The potential sites of phosphorylation are highlighted in red.

Fig.2. Activation and desensitization of PAR1. Thrombin (α -Th) binds to and cleaves the amino-terminal exodomain of PAR1 thereby generating a new amino terminus that acts as a tethered ligand by binding to the second extracellular loop of the receptor to cause transmembrane signaling. Activated PAR1 couples to multiple G protein subtypes including G_q , G_i and $G_{12/13}$ even in the same cell. Activated PAR1 signaling is then rapidly terminated by both GRK-mediated phosphorylation and binding of β arrestins. The C-tail is the major site for GRK phosphorylation, however, the receptor domain that mediates β arrestin binding is not known. Moreover, the relative contribution of phosphorylation *versus* β arrestin binding to the uncoupling of PAR1 from G_q signaling is not known. It has also not been determined how PAR1 uncouples from G_i and $G_{12/13}$ signaling. The function of GRKs and β arrestins in the termination of signaling by other PARs is not known.

Fig.3. Recruitment of activated PAR1 to clathrin-coated pits. Activated PAR1 rapidly colocalizes with clathrin and transferrin receptor (TfnR) in punctate-like structures at the plasma membrane (Trejo et al. 2000). PAR1 is internalized via a clathrin- and dynamin-dependent pathway in many cell types. Although phosphorylation of activated PAR1 promotes internalization from the cell surface, arrestins are not essential for this process. A highly conserved tyrosine based motif YSIL is present in the C-tail of PAR1 and appears to function in trafficking of the receptor. The adaptor protein complex AP-2 binds to tyrosine based motifs in other membrane receptors such as the TfnR and facilitates recruitment to clathrin coated pits. It is not known whether AP-2 functions in PAR1 internalization.

Fig. 1

PAR1 . . YASSECQRYVYYSI LCCKESSDPSSYNSSGQLMASKMDTCSSNLNNSI YKLLT
PAR2 . . FVSHDFRDHAKNALLCRSVRTVKQMQLVSLTSKKHSRKSSSYSSSSTTVKTSY
PAR3 . . LMSKTRNHSTAYLTK
PAR4 . . YVSAEFRDKVRAGLFQRS PGDTVASKAS AEGGSRGMGTHSLLQ

Fig. 2

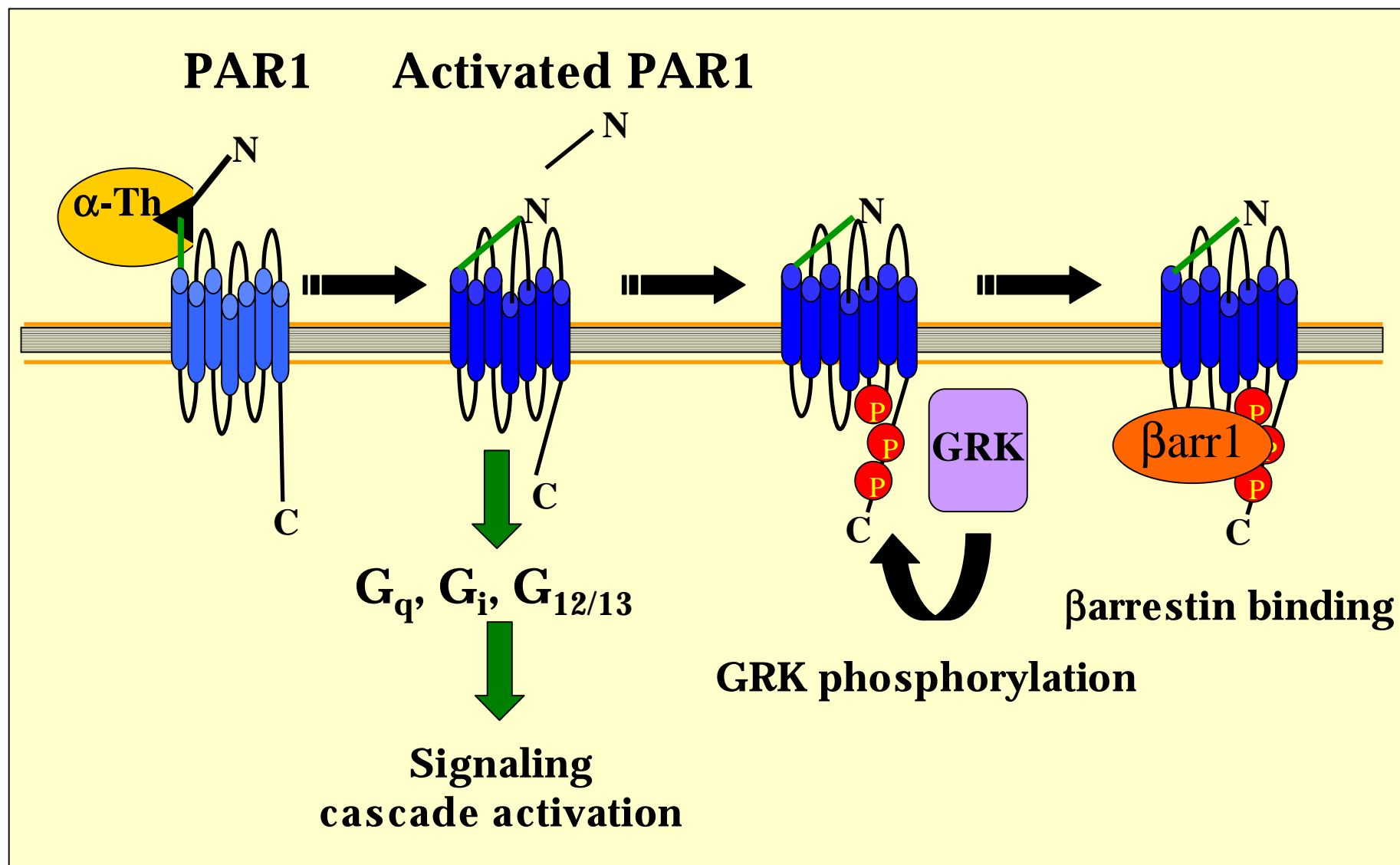


Fig. 3

