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THE CARDIOVASCULAR ACTIONS OF PROTEASE-ACTIVATED RECEPTORS

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ABBREVIATIONS:
Anion-binding recognition exosite 1: ABE1
Extracellular loop: ECL
G protein-coupled receptor: GPCR
Matrix metalloproteinase: MMP
Phospholipase C: PLC
Protease-activated receptors: PARs

Protease-activated receptors (PARs) comprise a family of G protein coupled receptors with a unique proteolytic activation mechanism. PARs are activated by thrombin or other coagulation or inflammatory proteases formed at sites of tissue injury. PARs play a particularly important role in pathogenesis of clinical disorders characterized by chronic inflammation or smoldering activation of the coagulation cascade. Individual PARs have been linked to the regulation of a broad range of cellular functions. Recent studies identify PAR family members in the vasculature (including within atherosclerotic lesions) and in the heart. Here, PAR-triggered responses contribute to vasoregulation and influence cardiac electrical and mechanical activity. PAR activation also is linked to structural remodeling of the vasculature and the myocardium. This review focuses on the cardiovascular actions of PARs that play a role in normal cardiovascular physiology and are likely to contribute to cardiovascular diseases.

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INTRODUCTION

Thrombin was first identified as a trypsin-like serine protease produced at sites of vessel injury or tissue damage, which plays a key role in blood coagulation by converting fibrinogen to fibrin (the fibrous matrix of blood clots). However, early studies exposed an additional effect of thrombin to promote platelet aggregation, even in the absence of any other coagulation factors. The molecular basis for thrombin's cellular actions (that are distinct from its role in clot formation) were first elucidated by the Coughlin laboratory with the cloning of PAR-1 in 1991 (Coughlin, 2000). Subsequent research identified PAR-1 as a prototype of a family of related PARs that have important non-hemostatic functions in development, play a role in tumor biology, and orchestrate a series of highly regulated responses that are integral to the inflammatory response and vital for normal tissue repair. The broad scope of cellular processes regulated by PARs is evident from the recent excellent reviews that consider roles for PARs in inflammation and wound healing, vasoregulation, angiogenesis, atherosclerosis, gastrointestinal disorders, pain perception by sensory neurons, airway hyperreactivity, and inflammatory pulmonary diseases (Vergnolle *et al.*, 2001;Vergnolle, 2000;Major *et al.*, 2003;Macfarlane *et al.*, 2001;Ossovskaya and Bunnett, 2004). PARs also exert a wide range of cardiovascular actions. Most of the published literature has focused on PAR-mediated action in platelets and the vasculature. Here, PARs are critical for normal hemostasis and contribute to the pathogenesis of vascular disorders characterized by chronic inflammation or smoldering activation of the coagulation cascade (including vascular atherosclerosis). However, there is recent evidence that PARs also exert direct effects on the heart that lead to changes in contractile performance and structural remodeling of ventricular cardiomyocytes. This review briefly summarizes the

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pharmacologic properties of the four known PAR family members before focusing on recent literature that explores the cardiovascular consequences of PAR activation.

PAR STRUCTURE AND ACTIVATION MECHANISMS

PAR-1

PAR-1 is the prototype for the family of G protein-coupled heptahelical receptors (GPCRs) that are activated via limited N-terminal proteolysis by serine proteases (Coughlin, 2000). Thrombin (the physiologic activator of PAR-1) docks to two sites on the PAR-1 N-terminus. The initial interaction is between the anion-binding recognition site of thrombin (anion-binding exosite I, or ABE1) and a negatively charged hirudin-like surface on PAR-1 ($DK^{51}YEPF^{55}$, Figs 1 and 2). This high-affinity interaction is believed to induce a conformational change that facilitates thrombin binding (via its catalytic site) to the PAR-1 cleavage sequence ($LDPR^{41}\downarrow S^{42}FLLRN$), an interaction energetically favored when the P_2 and/or P_4 positions of the P_4-P_1 sequence is occupied by proline residues (Jacques and Kuliopulos, 2003). PAR-1 cleavage exposes a new N-terminus, beginning with SFLLRN (human sequence) that acts as a tethered peptide ligand.

The proteolytic mechanism for PAR-1 activation can be bypassed entirely with a synthetic peptide that corresponds to the newly exposed N-terminal tethered ligand sequence. However, PAR-1 activation requires relatively high concentrations of soluble agonist peptide (AP; 10-20 μM); in contrast, picomolar thrombin is sufficient to proteolytically activate PAR-1 (Table 1). These differences generally have been attributed to the lack of a membrane tether; soluble APs need not assume the correct orientation relative to the ligand recognition sites and they also are likely to be more susceptible to the actions of degradative enzymes.

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Current models hold that PAR-1 activation results from [1] a docking interaction between the basic arginine residue at position 5 of the PAR-1-AP and a conserved glutamic acid in ECL2 and [2] an additional interaction between the tethered ligand aromatic Phe^{43} ring and the Ser^{89} side chain in the P^{85}AFIS^{89} sequence at the C-terminus of PAR-1's exodomain (Figs 1 and 2). However, there also is evidence that the precise docking sites for (or activation mechanisms triggered by) soluble and tethered ligands may differ. While these differences in surface contact points for soluble and tethered ligands (in the context of the relatively unfavorable energetics for PAR-1 activation by soluble ligands) might be predicted to hinder efforts to develop therapeutically useful small molecule PAR-1 agonists, early screens identified BMS 200261 as a potent PAR-1 agonist. BMS 200261's limitation is that it also exerts agonist activity at PAR-2, an observation not altogether surprising given the high degree of sequence homology between the ECL2 (putative tethered ligand-binding) sequences of PAR-1 and PAR-2 (Fig 3) as well as evidence that the human PAR-1 tethered ligand sequence SFLLRN is a potent agonist at PAR-2. PAR-1-selective activation has been accomplished with the *Xenopus* PAR-1 tethered ligand sequence (TFLLRN, which carries a position 1 Ser→Thr substitution). Similarly, RWJ-56110 has been developed as a peptidomimetic PAR-1-selective antagonist that effectively blocks PAR-1 activation (by either thrombin or SFLLRN) and exerts anti-restenotic activity in several angioplasty models ((Major *et al.*, 2003), Table 1).

PARs are endowed with two unique regulatory features as a result of their distinctive proteolytic activation mechanism. First, PAR-1 need not be selective for thrombin; rather, PAR-1 is activated by any serine protease that cleaves the N-terminus Arg^{41}-Ser^{42} bond (including coagulation factors upstream of thrombin such as Factor VIIa and Factor Xa and the anticoagulant activated protein C). Second, PAR-1 cleavage need not lead to receptor activation.

Rather, nonproductive cleavage events at sites that amputate the tethered ligand sequence render PAR-1 unresponsive to subsequent proteolytic activation (although PAR-1 cleaved in this manner generally remains otherwise structurally intact and fully responsive to SFLLRN). It has been speculated that nonproductive cleavage events fulfill a particularly important role (in addition to the traditional phosphorylation/internalization mechanisms for receptor desensitization) to terminate signaling by activated PARs, which irreversibly carry their tethered ligand. Indeed, a recent study linked PAR-1 activation (or activation of its downstream effector, protein kinase C) to regulated PAR-1 N-terminal exodomain shedding (although the physiologic importance of this process as a mechanism to terminate/modulate thrombin-dependent PAR-1 activation has not yet been established (Ludeman *et al.*, 2004)). Cathepsin G (a protease released from activated neutrophils at sites of injury and inflammation) is another example of a protease capable of amputating the PAR-1 tethered ligand. While cathepsin G can cleave the R_{41}-S_{42} bond (i.e., activate PAR-1) in heterologous overexpression systems, nonproductive cleavage events that amputate the tethered ligand appear to predominate in the physiologic context (Molino *et al.*, 1995). Finally, plasmin (an enzyme that is released from inactive precursor plasminogen by thrombolytics such as tissue plasminogen activator and plays an important role to cleave fibrin and dissolve clots) disables PAR-1 by cleaving the exodomain at one or more basic arginine/lysine residues (Fig 1, (Jacques and Kuliopulos, 2003)). Of note, most non-productive cleavage events have been identified for the human PAR-1 sequence; species-dependent differences in primary amino acid sequences (or glycosylation patterns) could influence this process and only infrequently have been considered.

PAR-2

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PAR-2 is a trypsin-activated receptor (i.e., the only PAR not effectively cleaved by thrombin) which is detected in a wide range of tissues; PAR-2 is particularly abundant in tissues exposed to the extracellular environment (i.e., the gastrointestinal tract and airways). Like PAR-1, PAR-2 is activated via limited proteolysis of its N-terminal exodomain or by a synthetic peptide corresponding to the first 6 amino acids of the newly exposed N-terminus of the cleaved receptor (SKGR↓SLIGRL, Table 1). Initial structure-activity relationship studies identified a functionally important charge-charge interaction between the basic position 5 residue in the PAR2-AP and a conserved ECL2 glutamic acid residue, analogous to the mechanism described for PAR-1. However, more recent mutagenesis studies reveal striking differences in the structure-activity relationships for soluble and tethered ligands, raising important questions regarding the nature of the tethered ligand domain-ECL-2 docking interaction (Ossovskaya and Bunnett, 2004). A simple intramolecular activation model also does not explain the unusual pharmacology of a recently described human PAR-2 polymorphic variant (PAR-2-F240S, involving a Phe→Ser mutation at position 240 of ECL2; Fig 3 and Table 2 (Compton *et al.*, 2000)). Compared to WT-PAR-2, PAR-2-F240S displays markedly reduced sensitivity to trypsin, impaired activation by rodent and human PAR-2-AP sequences (SLIGRL and SLIGKV, respectively), but ~4-fold increased sensitivity to the PAR-2-selective agonist tc-LIGRLO-NH_2. TLIGRL (a PAR-2-AP sequence with a position 1 Ser→Thr substitution) also is relatively selective for PAR-2-F240S; TLIGRL is a very weak agonist for WT-PAR-2. Finally, SFLLRN (a PAR-1-AP) exhibits similar potency at WT-PAR-2 and PAR-2-F240S, whereas TFLLR-NH_2 (the Ser→Thr substituted PAR-1-AP) and tc-YGPKF (a PAR-4-AP derivative) selectively activate PAR-2-F240S (not WT-PAR-2). The altered pharmacology of the PAR-2-F240S variant raises serious questions regarding the structure of endogenous PAR-2 peptide ligands in tissues

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and emphasizes the importance of considering polymorphisms in the design and clinical application of PAR-directed pharmaceuticals. In this regard, 2-furoylated (2f) derivatives of the native PAR-2-AP have been identified as effective *in vivo* PAR-2 agonists. 2f-LIGRL-NH_2 is the most potent furoylated-PAR-2-AP compound synthesized to date. It displays high intrinsic agonist activity and resists degradation by aminopeptidases ((Kawabata *et al.*, 2004), although its activity at the PAR-2-F240S polymorphic variant has not been considered).

Pancreatic trypsin is presumed to be the physiologic activator of PAR-2 in the intestinal lumen; trypsin (prematurely released from trypsinogen) also is presumed to activate PAR-2 in the inflamed pancreas. At sites not exposed to pancreatic trypsin, PAR-2 cleavage has been attributed to [1] extrapancreatic forms of trypsin, [2] tryptase, a serine protease that selectively activates PAR-2 (not PAR-1) and is released in high concentrations by degranulating mast cells, [3] membrane-type serine protease-1, a transmembrane protein that contains an extracellular protease domain and is coexpressed with PAR-2 in certain tissues, [4] coagulation proteases upstream of thrombin such as Factors VIIa and Xa, [5] airborne allergens (such as the dust mite serine proteases Der 3 and Der 9) that cleave PAR-2 on airway epithelial cells, leading to the release of proinflammatory mediators and airway hypersensitivity, and [6] proteases elaborated by bacterial pathogens such as *Porphyromonas gingivalis, Pseudomonas aeruginosa*, or *Legionelle pneumophila*. Of interest, *P. gingivalis* is the major causative agent of adult periodontal disease; it is reported to escape into the bloodstream and infect atherosclerotic plaques during routine oral hygiene procedures in patients with severe periodontal disease. The observation that *P. gingivalis* releases a protease that cleaves PAR-2 may explain the epidemiologic link between periodontal disease and increased cardiovascular risk. Collectively, the rather eclectic list of proteases that cleave PAR-2 has been taken to suggest that PAR-2 has

evolved its unique pharmacology to sample the extracellular environment at defensive barriers (such as the gastrointestinal tract and airways), detect foreign proteinases (released by ingested bacterial pathogens or inhaled irritants), and trigger primary inflammatory responses. In keeping with this concept, PAR-2 expression is upregulated by pro-inflammatory mediators (such as TNF α and IL-1); PAR-2 influences the perception of painful stimuli and plays a critical role in various acute and chronic inflammatory processes (Vergnolle *et al.*, 2001).

PAR-3/PAR-4

PAR-3 and PAR-4 are the most recently cloned 'thrombin receptors'. Both contain N-terminal serine protease cleavage sites, but their pharmacologic properties and functions are quite different. PAR-3 contains an N-terminal hirudin-like high-affinity thrombin-binding domain (FEEFP, analogous to the site on PAR-1, Fig 2). However, PAR-3 is the only known PAR not activated by a synthetic peptide corresponding to its putative tethered ligand. A detailed structural analysis of PAR-3 provides a clue to its distinct role in thrombin signaling. Unlike PAR-1 and PAR-4 (which contain P_2 position prolines that optimize their structural specificity for thrombin), mPAR-3 lacks a proline residue in the P_1-P_4 sequence (L^{34}TIK) and is a relatively poor thrombin substrate. hPAR-3 has a P_3 proline that actually sterically hinders its interaction with (and cleavage by) thrombin. These properties of PAR-3 make sense in the context of the current model that assigns PAR-3 a function as a co-receptor for PAR-4 in mouse platelets.

PAR-4 is activated via thrombin-dependent cleavage of the PAPR↓GYPGQV site or by synthetic peptides corresponding to the tethered ligand sequence exposed following thrombin cleavage. However, PAR-4 activation requires relatively high thrombin concentrations (~50-fold higher than the thrombin concentrations required to activate PAR-1), presumably because PAR-4

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lacks a hirudin-like high-affinity thrombin-binding domain (Fig 2). The AP concentrations required to activate PAR-4 also are substantially higher than those required for PAR-1 or PAR-2 activation by their cognate APs. However, agonist potency is substantially improved - without loosing specificity for PAR-4 – by substituting an alanine at position 1 in the AP sequence (AYPGKF, Table 1).

PAR-4 responses tend to be slow in onset and sustained, relative to the rapid and transient responses typically elicited by PAR-1. The relatively slow activation kinetics for PAR-4 are believed to be the direct consequence of the relatively slow rate of PAR-4 cleavage by thrombin. Current models suggest that the coexpression of multiple thrombin-sensitive PARs with distinct pharmacologic properties allows for graded responses to a wide range of thrombin concentrations and/or thrombin-dependent responses that follow different tempos, involve distinct intracellular effectors, or are localized to different membrane subdomains. As noted, multiple PARs also could enable responses a wide repertoire of proteases (in addition to thrombin). Indeed, PAR-4 has variably been characterized as a cellular receptor for trypsin and plasmin (Sambrano *et al.*, 2000;Quinton *et al.*, 2004). In the case of plasmin, the kinetics of PAR-4 cleavage are relatively slow. However, the generation of plasmin for protracted intervals (the goal of treatment for stroke or deep vein thrombosis) could result in the slow/indolent activation of PAR-4 and a paradoxical increase in platelet activation and thrombus formation. Finally, PAR-4 also has been reported to mediate the cellular actions of cathepsin G in an oocyte expression system (Sambrano *et al.*, 2000), although subsequent studies failed to detect PAR-4-mediated actions of cathepsin G in murine platelets or thrombin-responsive PAR-$1^{-/-}$ fibroblasts that overexpress PAR-4 (see below, (Sabri *et al.*, 2003a)). These discrepant results could suggest

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that PAR-4 cleavage by cathepsin G might be influenced by factors such as species differences in primary amino acid sequence or posttranslational modification (which deserve closer study).

CARDIOVASCULAR ACTIONS OF PARS

PAR ACTIONS IN THE VASCULATURE

The critical role of PAR-1 in vascular events was exposed when the PAR-1 gene was disrupted in mice. PAR-$1^{-/-}$ mice display a partial embryonic lethal phenotype. ~50% of PAR-$1^{-/-}$ embryos succumb to a fatal bleeding event between E9.5 and E12.5; the remainder show no obvious phenotype. Of note, a molecular strategy that drives PAR-1 expression only in endothelial cells (using the endothelial-specific TIE2 promoter-enhancer) is sufficient to rescue this phenotype, providing strong evidence that PAR-1 is required for normal vascular development in the embryo (Griffin *et al.*, 2001). Consistent with the role of PAR-1 in embryonic blood vessel growth and differentiation, thrombin signaling via PAR-1 also has been implicated in tumor neovascularization.

PARs also regulate vascular tone. A number of laboratories have described PAR-triggered cardiovascular responses in intact animal models. In rodents, PAR-1-AP infusion leads to a biphasic blood pressure response; a rapid/transient hypotensive response (mediated by NO) is followed by a more-sustained increase in blood pressure (that is presumed to reflect direct activation of PAR-1 on vascular smooth muscle cells). In contrast, PAR-2-AP infusion only induces hypotension in these models (Cicala *et al.*, 1999). The mechanism(s) for PAR-dependent vasoregulation have been explored largely using *in vitro* preparations of precontracted vessels. Here, PAR-1 and PAR-2 both induce vasorelaxation via an endothelial-dependent mechanism that generally is attributed to an NO-dependent process in large vessels and an NO-

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independent process (that in many cases involves a cyclooxygenase product) in smaller caliber vessels. In endothelium-denuded vessels, PAR-1 induces a contractile response via an action at the underlying smooth muscle layer. These PAR-dependent actions generally are assumed to gain importance as mechanisms that regulate local blood flow at sites of tissue damage, since PAR-$1^{-/-}$ and PAR-$2^{-/-}$ mice exhibit no obvious abnormalities in blood pressure regulation (at least at baseline, under unstressed conditions). However, there is recent evidence that PAR-2 induces arterial and venous dilatation *in vivo* in healthy humans and lipopolysaccharide (which upregulates PAR-2 expression) can markedly sensitize animals to the *in vivo* hypotensive effects of PAR-2 agonists (Robin *et al.*, 2003;Cicala *et al.*, 1999). These results suggest that PAR-2 activation could contribute to blood pressure dysregulation during sepsis, endotoxemia, or other states associated with serine protease activation.

PARs also participate in the response to vascular injury, a process most effectively examined in PAR-$1^{-/-}$ mice (where PAR-1-mediated actions of thrombin in the vasculature can be distinguished from the PAR-3/PAR-4-mediated actions of thrombin in platelets). PAR-$1^{-/-}$ mice exhibit reduced neointimal proliferation and restenosis when subjected to various models of vascular injury (balloon injury, endothelial denudation), suggesting that PAR-1 contributes to vascular injury responses and restenosis. Vascular remodeling has been viewed as the consequence of a series of highly orchestrated PAR-1 triggered responses. PAR-1 alters junctional complexes between endothelial cells, leading to increased monolayer permeability and the extravasation of plasma proteins. PAR-1 recruits platelets and leukocytes to injured surfaces by inducing the expression of inflammatory cytokines and adhesion molecules and increasing the production of PAF, prostenoids and nitric oxide. PAR-1 promotes vascular remodeling by stimulating vascular endothelial and smooth muscle cell proliferation (directly, and indirectly via

the production of growth factors) as well as by promoting the synthesis and release of matrix proteins and matrix degrading metalloproteinases. Of note, PAR-2 mimics some (but not all) of PAR-1's cellular actions. While PAR-2 does not mimic the *in vitro* effect of PAR-1 to increase endothelial monolayer permeability, PAR-2 stimulates vascular endothelial cell mitogenesis *in vitro* and it exerts a proangiogenic action (which is not accompanied by significant inflammatory changes) in *in vivo* normoperfused (nonischemic) skeletal muscles (Mirza *et al.*, 1996;Milia *et al.*, 2002). PAR-2 also is reported to potentiate reparative angiogenesis and enhance limb salvage in a hind limb ischemia model (Milia *et al.*, 2002). These proangiogenic actions of PAR-2 would be amplified in an ischemic milieu, where increased endothelial cell PAR-2 expression and the accumulation of PAR-2-bearing leukocytes would lead to the release of proangiogenic substances (Milia *et al.*, 2002). Finally, there is recent evidence that PAR-4 may play an ancillary role, with PAR-1, in thrombin-dependent signaling events in the vascular endothelium in mice (Kataoka *et al.*, 2003). The clinical relevance of this observation is uncertain, as PAR-4 function appears to be highly species- and/or model-specific; PAR-4 responses generally have not been detected in human endothelial cells (Kataoka *et al.*, 2003;O'Brien *et al.*, 2000). Collectively, these results implicate PARs as regulators of events that influence the evolution of atherosclerosis, ischemic tissue injury, and tumor angiogenesis.

PAR ACTIONS IN THE HEART

PAR-1 ACTIONS IN CARDIOMYOCYTES

PAR-1 activation by thrombin triggers a range of signaling events in cardiomyocytes that lead to rapid changes in electrical/mechanical function and could contribute to the genesis of the electrophysiologic derangements observed in the setting of myocardial ischemia and infarction.

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Thrombin increases spontaneous automaticity and/or elevates Ca_i in a range of cardiomyocyte preparations (Jiang *et al.*, 1996;Jiang *et al.*, 1998;Chien *et al.*, 1990;Albitz *et al.*, 1992). Thrombin also prolongs the action potential duration and increases cesium-induced early afterdepolarizations in isolated canine Purkinje fibers; thrombin induces proarrhythmic events during early reperfusion in intact adult rat hearts (Steinberg *et al.*, 1991;Jacobsen *et al.*, 1996;Woodcock *et al.*, 1998). Many of thrombin's proarrhythmic effects were described prior to the cloning of PAR-1, and hence did not consider the role of PAR-1. However, recent studies implicate PAR-1 in the IP_3-dependent proarrhythmic effects of thrombin during early reperfusion in rat hearts (Jacobsen *et al.*, 1996). PAR-1 activation also has been linked to a DAG/PKC pathway that activates Na^+-H^+ exchange and increases contractile performance in adult rat cardiomyocytes (Yasutake *et al.*, 1996). However, some thrombin-induced ionic derangements can not be attributed to phospholipid-derived second messenger molecules. For example, the effect of thrombin to mobilize intracellular calcium in neonatal rat cardiomyocytes and CHO cells is not blocked by a phospholipase C inhibitor; it can not be attributed to the conventional IP_3-dependent mechanism (Jiang *et al.*, 1996).

It may be no accident that most cardiac actions of PAR-1 have been described in intact tissue preparations or cardiomyocyte cultures. While we and others have linked PAR-1 activation to an increased contractile response adult cardiomyocytes acutely isolated from the intact ventricle (Yasutake *et al.*, 1996;Jiang *et al.*, 1998), PAR-1 responses typically are detected only at rather high AP concentrations in this preparation (an order of magnitude higher than the PAR-1-AP concentrations required to activate PAR-1 in other preparations). The molecular basis for these differences in PAR-1 responsiveness is suggested by recent studies of another GPCR; we recently demonstrated that standard enzyme-based cardiomyocyte isolation protocols

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lead to limited proteolysis of β-adrenergic receptors (β-ARs, (Rybin *et al.*, 2003)). Limited proteolysis would activate, desensitize, and/or disable PAR-1 (and render cells hyporesponsive to SFLLRN and unresponsive to thrombin). In support of this formulation, low concentrations of SFLLRN (which are subthreshold in acutely isolated adult cardiomyocytes) increase the force of isometric contraction in intact adult rat papillary muscles (Jiang *et al.*, 1998).

In keeping with PAR-1's actions as a potent mitogen for cells that maintain proliferative potential (such as vascular smooth muscle cells or fibroblasts), chronic/persistent activation of PAR-1 has been linked to a series of morphologic and molecular changes that are characteristic of the cardiomyocyte hypertrophic growth program; PAR-1 agonists increase protein content and cell size, increase sarcomeric organization, and induce early immediate gene expression. Of note, PAR-1 agonists increase cell length, but promote only a relatively minor increase in cell width; this morphology is characteristic of volume-overload dilated cardiac hypertrophy (Sabri *et al.*, 2000). In contrast, α_1-AR agonists induce a uniform increase cell dimension (cell length and width), which is more akin to the changes observed in the setting of pressure-overload hypertrophy. The signaling mechanisms that distinguish these morphologically distinct forms of cardiac hypertrophy (which carry different prognoses when encountered in clinical practice) are not obvious, since PAR-1 and α_1-ARs activate many common effector pathways (PLC, ERK, JNK, p38-MAPK, and AKT; Fig 4A). Nevertheless, some differences in the signaling molecules recruited by activated PAR-1 and α_1-AR (that could impact on the cardiac phenotype) have been identified. First, the α_1-AR growth response is attributable exclusively to pathways emanating from Gq. In contrast, PAR-1 promotes cardiac growth via dual pathways emanating from both Gq and Gi. This difference may be pertinent, as a genetic model of persistent Gi activation (due to G_i-coupled receptor overexpression) has been linked to a dilated form of cardiomyopathy in

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mice. The effect of PAR-1 to trigger a prominent increase in Ca_i (far in excess of the relatively minor calcium mobilizing effects induced by α_1-ARs (Jiang *et al.*, 1996;Sabri *et al.*, 2000)) also could contribute to the pathogenesis of a morphologically and functionally distinct form of cardiac hypertrophy. These and other mechanisms that might distinguish the hypertrophic signaling phenotypes induced by different GPCRs are the focus of ongoing research.

CARDIAC ACTIONS OF PAR-2

PAR-2 activates a spectrum of biochemical and functional responses that largely mimic cardiomyocyte activation by PAR-1 (including PLC, ERK, p38-MAPK, increased Ca_i, enhanced spontaneous automaticity, and elongated/dilated hypertrophy) in neonatal rat cardiomyocyte cultures (Sabri *et al.*, 2000). The relevant PAR-2-activating protease in the heart has not been identified. However, mast cell tryptase is a likely candidate, given reports that mast cells can be identified between muscle fibers in normal ventricles, mast cells are present in increased numbers in idiopathic and dilated cardiomyopathies (Patella *et al.*, 1998), and that tissue tryptase levels are elevated to levels that could potentially trigger PAR-2 signaling in certain cardiac syndromes (Patella *et al.*, 1998). Of note, the effect of tryptase to activate PAR-2 is variably detected in different cell types. Cell-specific differences in PAR-2 activation by tryptase has been attributed to an N-linked glycosylation 6 residues away from the hPAR-2 cleavage site (13 residues away from the rPAR-2 cleavage site); this glycosylation event appears to prevent PAR-2 activation by tryptase, but not trypsin (Compton *et al.*, 2002). This additional mechanism to regulate PAR-2 signaling should be considered in future studies of cardiac PAR-2 actions.

PAR-2 agonists enhance the efficiency of ischemic preconditioning, improve myocardial functional recovery, and decrease the incidence of ventricular arrhythmias in an *in vivo* ischemia-

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reperfusion cardiac injury model in rats (Napoli *et al.*, 2000). The cardioprotective effects of PAR-2 would not necessarily have been predicted based upon literature that casts PAR-2 as an activator of pro-inflammatory events in many non-cardiac tissues. However, the beneficial effects of PAR-2 have been attributed to PAR-2-dependent regulation of coronary vascular tone. PAR-2 agonists promote coronary vasodilation (via an endothelium-dependent, NO-independent mechanism) leading to improved perfusion of the compromised ventricle. The preserved PAR-2-dependent coronary vasodilatory response is in stark contrast to the reduced/absent responses to other endothelium-dependent vasodilators (such as acetylcholine and bradykinin) that characterize the endothelial dysfunction that develops during ischemia-reperfusion (McLean *et al.*, 2002). Upregulation of PAR-2 expression in the setting of ischemia-reperfusion and early atherosclerotic lesions has been offered as an explanation for the preserved PAR-2-induced vasodilatory response. Hence, PAR-2 represents a promising therapeutic target to influence the pathogenesis of *in vivo* ischemia-reperfusion injuries.

CARDIAC ACTIONS OF PAR-4

PAR-4 was originally identified as a receptor that is expressed at low levels in the mouse heart; PAR-3 is not detected in the heart. However, recent studies localize PAR-4 mRNA to cardiomyocytes themselves (rather than cardiac fibroblasts) and identify cardiac actions for PAR-4 that are distinct from the cardiac actions of PAR-1 (Sabri *et al.*, 2003b). PAR-4 activation leads to a modest increase in PLC and ERK activity and a weak hypertrophic response (relative to the robust PLC/ERK responses triggered by agonists for PAR-1 or α_1-AR in this preparation, Fig 4A). This is surprising (and unexplained) as PAR-4 agonists couple to a robust PLC/ERK response in PAR-$1^{-/-}$ lung fibroblasts that heterologously overexpress PAR-4.

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However, PAR-4 induces a rather strong activation of a non-receptor Src tyrosine kinase-p38-MAPK cascade in cardiomyocytes. Studies in PAR-1$^{-/-}$ cardiomyocytes yield additional surprising evidence that the effect of thrombin to activate Src in cardiomyocytes is mediated exclusively by PAR-4 (not PAR-1, as might be assumed based upon previous literature of thrombin's actions in other cell types (Sabri *et al.*, 2003b)). Finally, studies in PAR-1$^{-/-}$ fibroblasts that heterologously overexpress PAR-4 show that PAR-4 couples to the activation of PLC, ERK, and p38-MAPK via a pathway that involves EGFR transactivation. These studies suggest that PAR-4 may play a more general role to link thrombin stimulation to the activation of growth regulatory pathways mediated by receptor and non-receptor tyrosine kinases.

The functional consequences of PAR-4 expression/activation in cardiomyocytes remain uncertain. We have speculated that PAR-4 plays only a minor role under normal physiological conditions, where the cardiac responses to thrombin are mediated primarily by PAR-1. However, pathologic conditions that render PAR-1 inactive (such as inflammation, and the elaboration of proteases that amputate PAR-1's N-terminal tethered ligand) might shift the balance of signaling by PAR-1 and PAR-4. Under these conditions, unopposed PAR-4 activation of the Src/p38-MAPK pathway might lead to more adverse functional outcomes.

PAR-1 ACTIONS IN CARDIAC FIBROBLASTS

Cardiac fibroblasts are a major cellular component of intact ventricular myocardial tissue. Cardiac fibroblast activation by GPCR agonists (generally angiotensin II and endothelin) has been linked to proliferation and the synthesis of matrix components, responses that are essential for normal scar formation at sites of myocardial injury/infarction. However, excessive cardiac fibroblast activation (in the setting of hypertension) can lead to diastolic stiffness and mechanical

failure; exuberant fibroblast-dependent synthesis of paracrine growth factors or matrix components also can lead to noncontractile fibrotic scars that disrupt the normal transmission of electrical impulses. Recent studies identify PAR-1 as a functionally important GPCR in cardiac fibroblasts; cardiac fibroblasts do not express PAR-2, PAR-3 or PAR-4. PAR-1 activates a spectrum of signaling responses (including PLC, calcium, ERK, p38-MAPK, and AKT) that lead to increased cardiac fibroblast proliferation (Sabri *et al.*, 2002).

The detailed signaling mechanisms activated by PAR-1 in cardiomyocytes and cardiac fibroblasts are quite distinct (Fig 4). Most notably, transactivation of EGFRs (or other receptor tyrosine kinase family members) plays little-to-no role in PAR-1 signaling to ERK and hypertrophy in cardiomyocytes. In contrast, non-receptor tyrosine kinases (including Src and Fyn) and the epidermal growth factor receptor (EGFR) play a pivotal role to link PAR-1 to the stimulation of ERK, p38-MAPK, and AKT, and increased DNA synthesis (as a measure of proliferation) in cardiac fibroblasts (Sabri *et al.*, 2002). These results emphasize the highly contextual, cell-specific nature of PAR-1 signaling in the heart. Another striking difference between PAR-1 signaling responses in cardiomyocytes and cardiac fibroblasts relates to AKT. PAR-1 agonists induce a very robust increase in AKT (via EGFR transactivation) in cardiac fibroblasts; in contrast, PAR-1 does not significantly transactivate EGFR family members and leads to only a very minor increase in AKT in cardiomyocytes (Sabri *et al.*, 2002). These studies are consistent with the notion that robust GPCR-dependent AKT activation is confined to cells (and GPCRs) that support the EGFR transactivation mechanism. Given the importance of AKT as a therapeutic target for heart failure, progress towards identifying the mechanisms that enable GPCR networking to EGFR transactivation (and AKT activation) would hold profound clinical significance.

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CARDIOMYOCYTE ACTIVATION BY PROTEASES OTHER THAN THROMBIN

The endogenous PAR activators in the heart remain uncertain. While cardiomyocyte PARs may be activated by thrombin in the setting of hemorrhagic infarction (where the endothelial barrier is broken and cardiomyocytes come into direct contact with blood-borne substances), most myocardial events are not accompanied by hemorrhage into the myocardium. As noted, mast cell degranulation might lead to the release of serine proteases such as tryptase and activation of PAR-2. The border zone adjacent to a myocardial infarction (an area characterized by intense interstitial inflammation and important local changes in gene expression, cardiomyocyte hypertrophy, contractile dysfunction, and apoptosis) might be another region prone to PAR signaling events. Based upon published evidence that PAR-4 can function as a cellular receptor for neutrophil-derived cathepsin G (in the context of evidence that cardiomyocyte express functional PAR-4), we recently considered whether cathepsin G exerts direct cardiac actions (Sabri *et al.*, 2003a). We identified cathepsin G as a potent cardiomyocyte agonist. Cathepsin G induces a spectrum of acute signaling responses (including activation of PLC, ERK and p38-MAPK, stimulation of AKT, and changes in contractile function) that in many respects mimic the cardiac actions of thrombin. However, detailed studies yielded several lines of evidence to argue that the cardiac actions of cathepsin G can not be attributed to the activation of any known PAR. First, we found that cathepsin G induces a similar increase in PLC and p38-MAPK activity in WT and PAR-$1^{-/-}$ cardiomyocytes; these results effectively exclude a role for PAR-1 in cardiac cathepsin G responses (although cathepsin G-dependent disabling cleavage events for both human and mouse PAR-1 sequences were identified (Sabri *et al.*, 2003a)). Second, we found that cathepsin G disrupts the sarcomeric structure and induces progressive loss of cell-cell and cell-matrix contacts and leads to cell rounding and detachment

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from underlying matrix (i.e., detachment-induced apoptosis or anoikis, (Sabri *et al.*, 2003a)). This morphology is strikingly different from the hypertrophic phenotype induced by conventional PAR agonists. However, it is quite reminiscent of the cathepsin G-induced changes reported in endothelial cell monolayers, where cathepsin G impairs monolayer barrier function and exposes the potentially thrombogenic underlying extracellular matrix (Iacoviello *et al.*, 1995). Third, cathepsin G activates executioner caspases which cleave survival signals (FAK and AKT) and sarcomeric proteins (troponin T) in cardiomyocytes; none of these events are observed in cells exposed to thrombin. Finally, we could not detect cathepsin G responses in PAR-$1^{-/-}$ fibroblasts that overexpress either PAR-1 or PAR-4 (and exhibit robust responses to thrombin). Collectively, these results raise serious doubts that cathepsin G's action in cardiomyocytes (and likely other adherent cell types) can be attributed to the activation of a known PAR. The precise cellular target(s) for cathepsin G's actions have not yet been identified. Of interest, cathepsin G has been reported to degrade matrix components (either directly or indirectly via the cleavage/activation of latent matrix-degrading metalloproteinases). The role of matrix metalloproteinases versus other potential targets for cathepsin G's actions is a focus of ongoing studies.

FUTURE CHALLENGES

Initial efforts to clone a thrombin receptor were fueled by the expectation that thrombin receptor antagonists could be used to interfere with thrombin's cellular actions, without increasing bleeding diathesis by inhibiting fibrin formation. The cloning of four PARs whose roles extend to a wide range of cellular processes has identified novel therapeutic targets for a variety of common clinical disorders. This extends to the heart, where PAR-1 and PAR-2

influence cardiomyocyte electrical and mechanical events and PAR-1, PAR-2, and PAR-4 promote cardiac structural remodeling (through actions in both the cardiomyocytes themselves as well as the supporting cardiac fibroblasts). Despite substantial progress towards understanding the activation mechanisms and functions of individual PARs in the heart, many important questions remain outstanding. The challenges for future research will be to identify the physiologically relevant proteases that activate (and disarm) cardiac PARs, determine whether the heart might be a source of endogenous peptide ligands that locally regulate PARs, determine whether PAR expression is influenced by the hypertrophic growth program or cardiac failure, explore roles for polymorphic variants of PARs as a disease modifiers, and develop more potent agonists and antagonists to regulate signaling by individual PAR family members (including in the *in vivo* context). Collectively, these types of studies will lay the groundwork to consider PARs as therapeutic targets in newer strategies to prevent and treat the functional and structural abnormalities that result from cardiac injury and inflammation.

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FOOTNOTES

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Figure 1. Schematic representation of the topology of PAR-1. Functionally important sequences in the N-terminal exodomain, such as the P_4-P_1 residues which influence thrombin binding/catalysis (blue), the tethered ligand sequence (green), the hirudin-like high affinity thrombin-binding site (pink), the acidic cluster (red), and the putative N-terminal exodomain ligand binding site (purple) are depicted. I^{88}, S^{89}, and L^{96} in the putative N-terminal exodomain ligand binding site (represented by gray filled circles) are sites where alanine substitution results in a profound defect in PAR-1 activation by SFLLRN (but not thrombin (Blackhart *et al.*, 2000)). The location of potential thrombin, APC/FXa, trypsin, cathepsin G, elastase, proteinase 3, and plasmin cleavage sites on PAR-1's N-terminal exodomain are indicated (Renesto *et al.*, 1997). Ser/Thr residues that are potential sites for GRK-mediated phosphorylation in the C-tail are highlighted by the filled red circles. A tyrosine-based YXXL sorting motif recently implicated in PAR-1 trafficking and internalization is highlighted in red circles.

Figure 2. Sequence alignment of thrombin receptor exodomains; numbering based upon human PAR-1 sequence. Functionally important sequences in the N-terminal exodomains of human (h), rat (r), and mouse (m) PAR-1 are illustrated as described in Fig 1. The conserved sequences in PAR-1, PAR-3, and hirudin are illustrated in pink. PAR-4 lacks this hirudin-like high-affinity thrombin binding site (that allosterically regulates thrombin cleavage at the catalytic site). To compensate, PAR-4 uses proline residues in the P_4-P_1 sequence to bind thrombin's catalytic site with high-affinity and the cluster of acidic residues in the middle of PAR-4's exodomain to slow the dissociation rate (and thereby increase the interaction time) of the thrombin-PAR-4 complex (Jacques and Kuliopulos, 2003).

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Figure 3. Sequence alignment of the conserved extracellular loop 2 region in the four known PARs. Residues that are identical in PAR-1, PAR-2 and PAR-3 (and in some cases PAR-4) are depicted in blue. Residues conserved in PAR-2 and PAR-3 (and in some cases PAR-4, but not PAR-1) are in yellow. The acidic glutamic acid that is believed to dock the tethered ligand position 5 arginine residue is in red. Potential N-linked glycosylation sites are highlighted in green. The location of the human PAR-2-F240S polymorphism is depicted in purple.

Figure 4. Schematics depicting the distinct cellular actions of PAR-1 and PAR-4 in cardiomyocytes (Panel A) and the PAR-1 signaling mechanisms involving EGFR transactivation in cardiac fibroblasts (Panel B). This schematic highlights important differences in the cellular actions of individual PARs in cardiomyocytes and the cell-specific differences in PAR-1 action in cardiomyocytes and cardiac fibroblasts. Cardiomyocytes co-express PAR-1 (which couples to the activation of phospholipase C, an increase in intracellular calcium, activation of ERK, p38-MAPK, and AKT, increased automaticity, and the hypertrophic growth responses) and PAR-4 (which activates a Src/p38-MAPK pathway, but has only a very minor effect on phospholipase C or ERK). EGFR transactivation plays little-to-no role in PAR-1 actions in cardiomyocytes (*Panel A*). In contrast, cardiac fibroblasts express PAR-1 (but no other known PARs) which activate ERK, p38-MAPK, and AKT via a pathway that involves Src family kinases and EGFR transactivation (*Panel B*).

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TABLE 1. STRUCTURAL AND FUNCTIONAL FEATURES OF PARS

	PAR-1	PAR-2	PAR-3	PAR-4
Primary Activating Protease (EC_{50})	Thrombin (50 pM)	Trypsin (1 nM) Tryptase (1 nM)	Thrombin (0.2 nM)	Thrombin (5nM) Trypsin (1 nM)
Other Activating Proteases	Trypsin FXa	FXa TF/FVIIa MT-SP1 Bacterial proteases Der P3 D9		Trypsin Plasmin Cathepsin G [a]
Inactivating Proteases	Cathepsin G Plasmin Proteinase 3 Elastase TACE-like MMP	Cathepsin G Plasmin Proteinase 3 Elastase		
Cleavage Sequence	LD**P**R ↓ SFLLRN VN**P**R ↓ SFFLRN	SKGR ↓ SLIGKV SKGR ↓ SLIGRL	L**P**IK ↓ TFRGAP LTIK ↓ SNGGP	**P**A**P**R ↓ YPGQV **PNP**R ↓ YPGKF
Tethered Ligand Sequence	SFLLRN (h) SFLLRN (m,r) TFRIFD (x)	SLIGKV (h) SLIGRL (m,r)	TFRGAP (h) SFNGGP (m) ?	GYPGQV (h) GYPGKF (m)
Hirudin-like sequence	Yes	No	Yes	No
Agonist peptides (generally as amides)	SFLLRN TFLLRN	SLIGKV SLIGRL SFLLRN tc-LIGRLO	None Known	GYPGKF AYPGKF tc-YGPKF tf-LIGRL
Antagonists	BMS 200261 RWJ-56110	BMS 200261		
Chromosome	5q13 (h) 13D2 (m)	5q13 (h) 13D2 (m)	5q13 (h) 13D2 (m)	19p12 (h) 8B3.3 (m)

A summary of the proteases that activate or inactive individual PARs, the sequences surrounding the proteolytic cleavage sites of each PAR family member, the human (h), mouse (m), rat (r), or *Xenopus* (x) tethered ligand sequences, currently available PAR antagonists, and the chromosomal localization of individual PAR family members. References and further details are provided in the text.

[a] Results examining the role of PAR-4 as a cellular cathepsin G receptor are inconsistent (see text).

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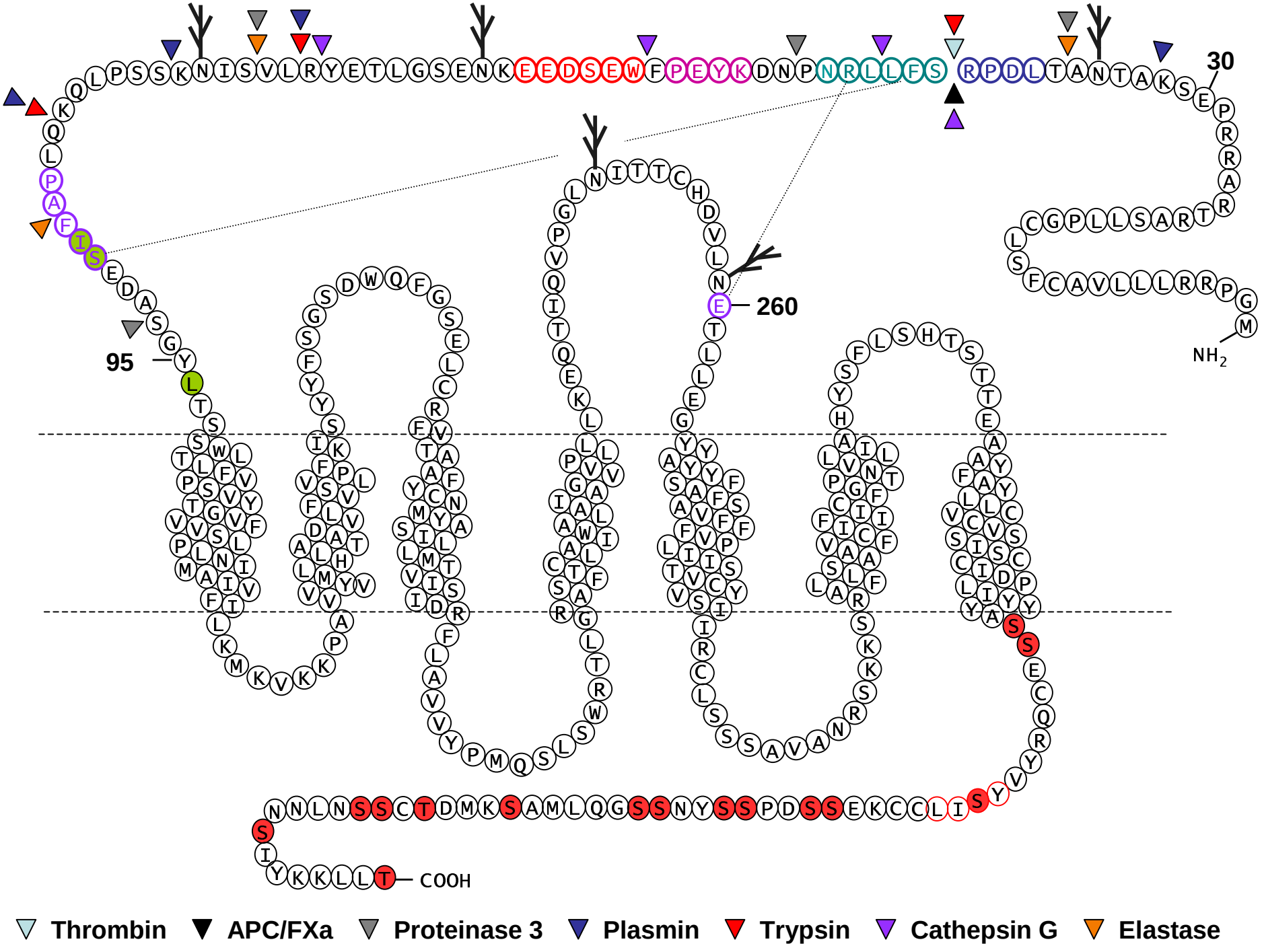
TABLE 2. DISTINCT PHARMACOLOGY OF PAR-2 POLYMORPHIC VARIANTS

	PAR-2-Phe^{240}	**PAR-2-Ser^{240}**
Allele Frequency	0.916	0.084
Activating Protease	trypsin	
Peptide Agonist	SLIGRL SLIGKV SFLLRN	tc-LIGRLO TLIGRL SFLLRN TFLLR tc-YGPKF

A summary of the distinct pharmacologic properties of the human polymorphic variants of PAR-2, based upon studies by Compton et al. that are described further in the text (Compton *et al.*, 2000).



THROMBIN RECEPTORS: *PAR-1, PAR-3, and PAR-4*

N-terminus: cleavage site, tethered ligand, putative ligand binding domain

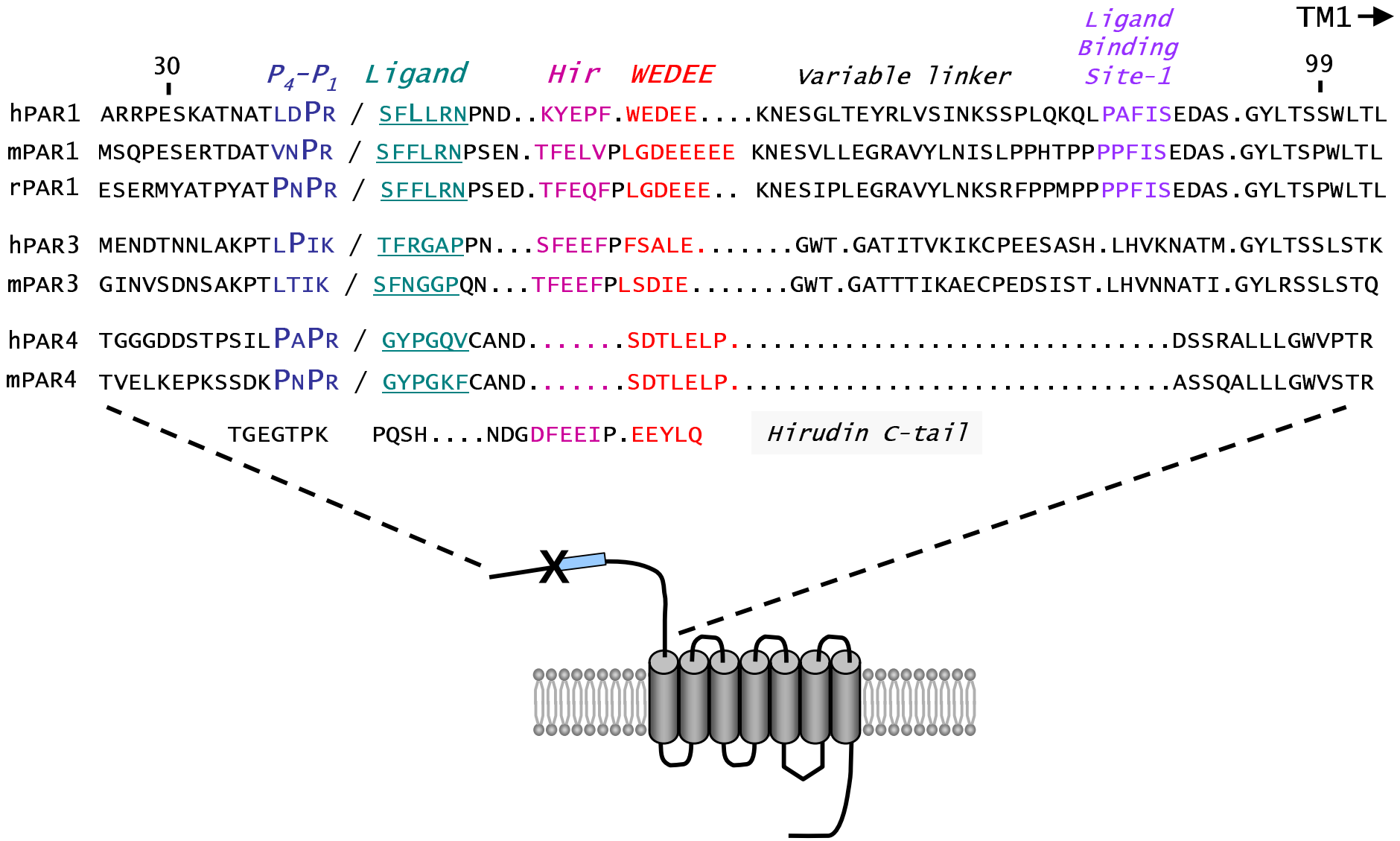


Figure 2

PAR Extracellular Loop 2 Sequences - ligand-binding doamin

hPAR-1 QTIQVPGLNITTCHDVLNETLLEG

hPAR-2 VKQTIFIPALNITTCHDVLPEQLLVGDM($F^{240}S$)NYFLS

hPAR-3 QTIFIPALNITTCHDVLPEQLLVG

hPAR-4 QTFRLARSDRVLCHDALPLDAQAS

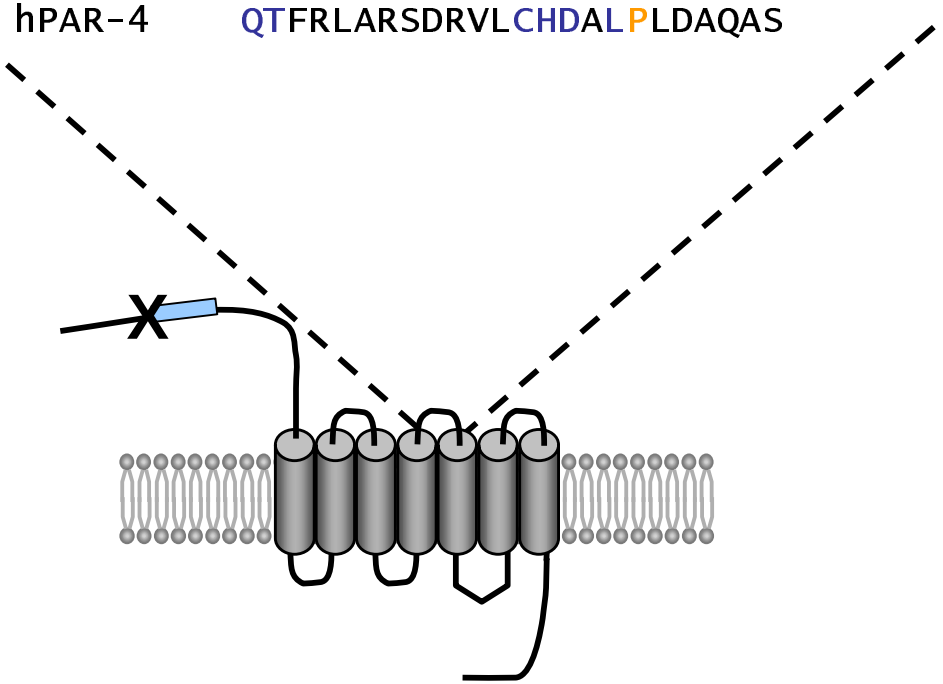


Figure 3

THROMBIN SIGNALING VIA PAR-1 AND PAR-4 IN CARDIOMYOCYTES

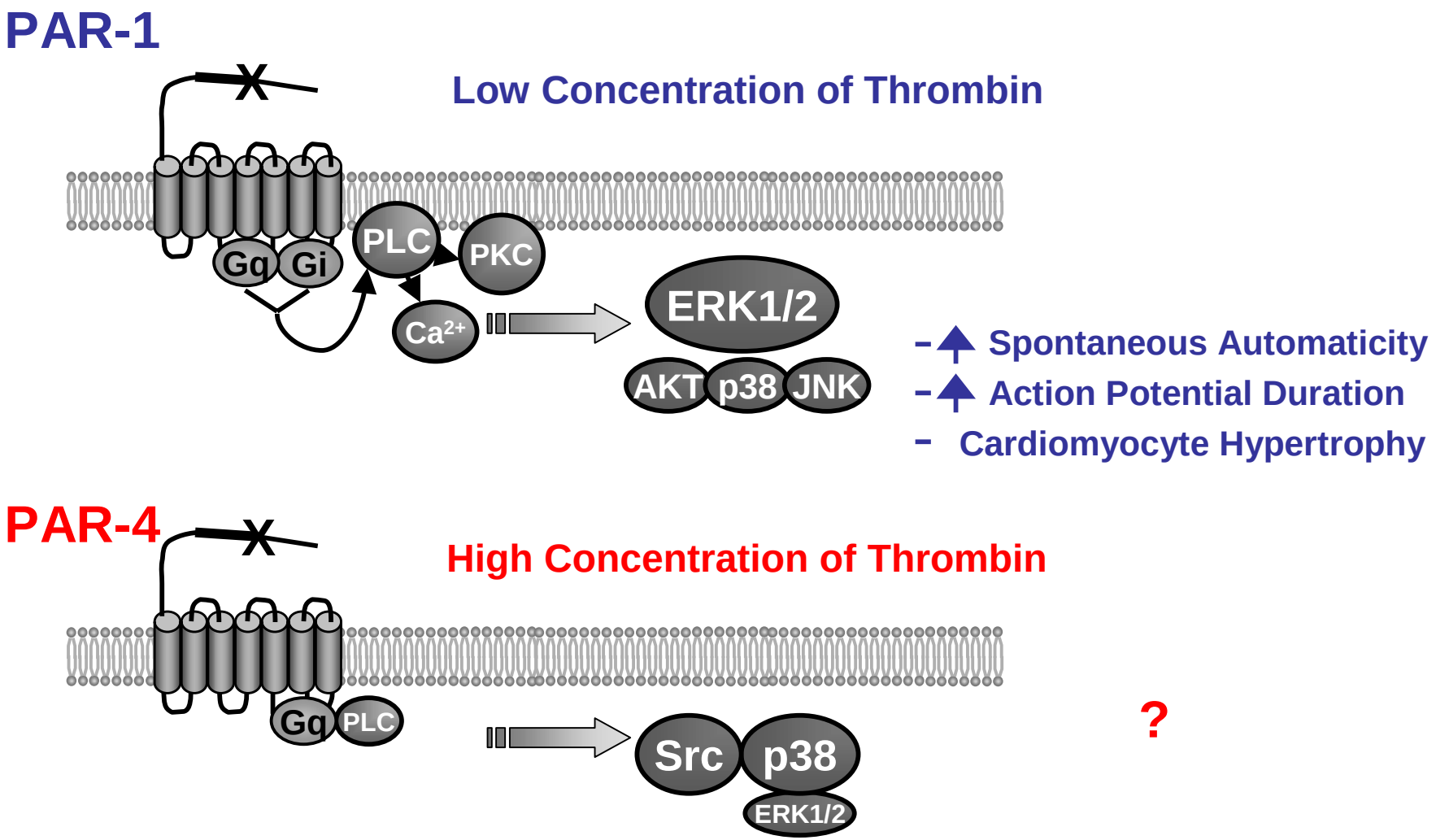


Figure 4A

Thrombin signaling via PAR-1 and EGFR Transactivation in Cardiac Fibroblasts

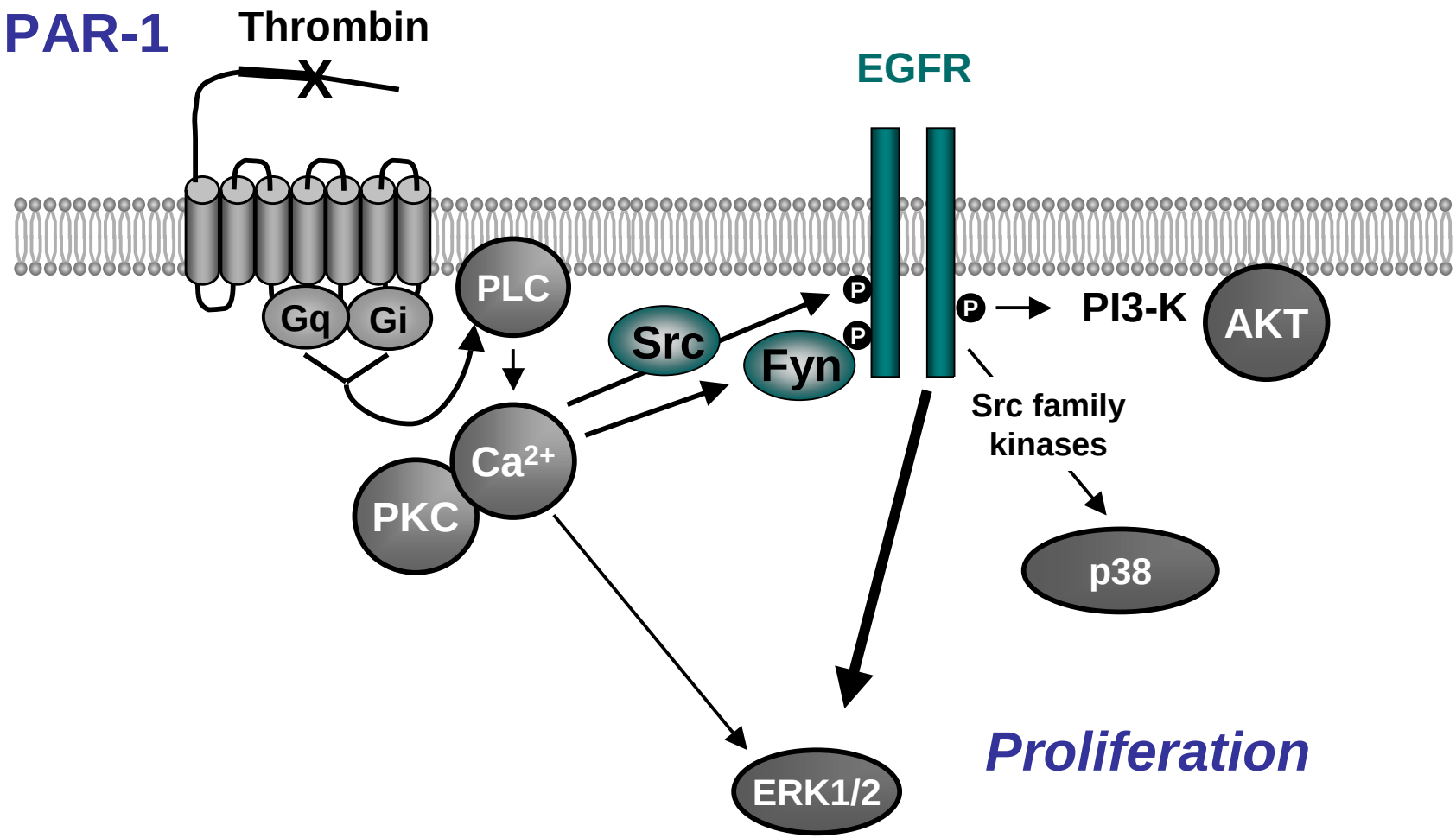


Figure 4B