Mechanisms of Protease-activated Receptor-4 Actions in Cardiomyocytes

ROLE OF Src TYROSINE KINASE*

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Protease-activated receptor (PAR-4) is a low affinity thrombin receptor with slow activation and desensitization kinetics relative to PAR-1. This study provides novel evidence that cardiomyocytes express functional PAR-4 whose signaling phenotype is distinct from PAR-1 in cardiomyocytes. AYPGKF, a modified PAR-4 agonist with increased potency at PAR-4, activates p38 mitogen-activated protein kinase but is a weak activator of phospholipase C, extracellular signal-regulated kinase, and cardiomyocyte hypertrophy; AYPGKF and thrombin, but not the PAR-1 agonist SFLLRN, activate Src. The observation that AYPGKF and thrombin activate Src in cardiomyocytes cultured from PAR-1−/− mice establishes that Src activation is via PAR-4 (and not PAR-1) in cardiomyocytes. Further studies implicate Src and epidermal growth factor receptor (EGFR) kinase activity in the PAR-4-dependent p38 mitogen-activated protein kinase signaling pathway. Thrombin phosphorylates EGFRs and ErbB2 via a PP1-sensitive pathway in PAR-1−/− cells that stably overexpress PAR-4; the Src-mediated pathway for EGFR/ErbB2 transactivation underlies the protracted phases of thrombin-dependent extracellular signal-regulated kinase activation in PAR-1−/− cells that overexpress PAR-4 and in cardiomyocytes. These studies identify a unique signaling phenotype for PAR-4 (relative to other cardiomyocyte G protein-coupled receptors) that is predicted to contribute to cardiac remodeling and influence the functional outcome at sites of cardiac inflammation.

Serine proteases regulate cell functions in large part by activating a family of seven transmembrane-domain spanning G protein-coupled receptors (GPCRs).1 PAR-1, the prototypical receptor for thrombin, is a ubiquitously expressed GPCR that is activated by cleavage of its extracellular N terminus to expose a new N-terminal sequence that binds intramolecularly and serves as a tethered ligand (1, 2). Since the initial cloning of PAR-1, three additional structurally homologous PARs have been identified; two newer PAR family members (PAR-3 and PAR-4) are activated by thrombin, whereas PAR-2 is activated as a result of limited proteolysis by trypsin, membrane-type serine protease-1 (a type II transmembrane protein with serine protease activity) or mast cell tryptase (not by thrombin (3, 4)).

Synthetic peptides that mimic the tethered ligand domains of PAR-1 (SFLLRN), PAR-2 (SLIGRL), and PAR-4 (GYPGKF), but not PAR-3, activate their cognate receptors independent of proteolysis (2). Detailed studies identify PAR-1 coupling to several heterotrimeric G protein family members (Gαs, Gαi, and G12/13) and thereby a host of intracellular response mechanisms that influence cell shape, growth, and differentiation. In contrast, knowledge of PAR-4 expression and function is still quite limited. In heterologous expression systems, PAR-4 mimics the actions of PAR-1 to activate phospholipase C and mobilize intracellular calcium. However, major differences between PAR-1 and PAR-4 in their sensitivity to activation by thrombin and kinetics of activation and desensitization have been identified. Specifically, PAR-1 is efficiently cleaved by low concentrations of thrombin, but PAR-4 activation requires much higher thrombin concentrations. PAR-4 activation and desensitization kinetics also are quite slow and sustained relative to the rapid and transient responses typically elicited by PAR-1 (5). Although these results suggest that PAR-1 and PAR-4 might not be functionally redundant, there is still scant information on distinct functional properties of PAR-1 and PAR-4 in native tissues.

The signaling properties of PAR-1 have been explored largely in platelets (where the actions of thrombin are critical for normal hemostasis and arterial thrombosis) and the vessel wall (where PAR-1 promotes changes in endothelial cell morphology leading to altered monolayer permeability and PAR-1 induces proliferation of vascular smooth muscle cells (2)). Recent studies indicate that cardiomyocytes represent an additional cardiovascular target for the actions of thrombin and related proteases. Cardiomyocytes cultured from neonatal rat ventricles co-express PAR-1 and PAR-2 (mRNA for PAR-3 is not detected), which stimulate phosphoinositide hydrolysis, activate the extracellular signal-regulated kinase (ERK) subfamily of mitogen-activated protein kinases (MAPKs), increase intracellular calcium, and promote cardiomyocyte hypertrophy (6–10). This study tests the hypothesis that cardiomyocytes also express PAR-4 and that PAR-4 activates mechanisms that contribute to cardiac remodeling in areas of cardiac injury and/or inflammation.
Preparation and Culture of Ventricular Myocytes—Cardiac myocytes were dissociated from the ventricles of outbred, PAR-1 knockout, or background strain C57BL/6 mice (embryonic day 18 (11)) by a trypsin digestion protocol that incorporates a differential attachment procedure to enrich for cardiac myocytes (6). Although the predigesting step effectually decreases fibroblast contamination, myocytes were subjected to 30 grays of X-rays on day 1 of culture to halt the proliferative potential of any residual contaminating fibroblasts (6). The myocytes were plated at a density of 0.5 × 10^6 cells/ml (2 ml/25-mm dish) and were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. For assays of ERK, p38-MAPK, or Src activation, the cells were serum-starved in 1:1 Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium for 24 h.

Reverse Transcraptase-Polymerase Chain Reaction—Total RNA was extracted using Trizol Reagent and was reverse transcribed to random primed cDNA with Superscript reverse transcriptase (Invitrogen). The PCR primers used for PAR-4 were P4Pan-U 5’-GCCAATGGGCGTG-GGCGCTGTTG-3’ and P4Pan-L 5’-GCCAGCGGATGAGGCGCCG-3’. The reactions were carried out in 50-µl reactions using Advantage KlenTaq polymerase (Clontech) with the indicated number of cycles consisting of a 30 s 94 °C denaturation followed by a 30 s 63.1 °C annealing with a 60 s elongation at 68 °C. The PCR primers used for β-actin were β-actin-PTP-U 5’-AGGCCAACCGAGGAAGATG-3’ and β-actin-PTP-L 5’-CTCGGCCGTGGTGTGAAGC-3’. The β-actin reactions were carried out in 25-µl reactions using Advantage KlenTaq polymerase (Clontech) for 25 cycles consisting of a 30 s 94 °C denaturation followed by a 30 s 60.4 °C annealing with a 30 s elongation at 68 °C. The amplified products were fractionated in 2% agarose gels and Southern transferred to Hybond N membrane (Amersham Biosciences). The blots were hybridized to the appropriate digoxigenin (DIG)-labeled nested primer probes; PANP4 PP-L 5’-CAGCGAGCAA-CACGTGAAATCATAGTGGCCCATAGAG-3’ for PAR-4, and actin PP-L 5’-TTGCCCACGTGTTGACCCGTGCCGCTTGTCATCA-C-3’ for β-actin. The specific PCR products were then visualized using DIG luminescent detection (Roche Molecular Biochemicals). Phosphoinositide Hydrolysis—The cardiomyocytes were incubated for 72 h with 3 µCi/ml [3H]myoinositol, washed, preincubated with 10 mM LiCl for 20 min, and then stimulated with agonists for the indicated intervals at room temperature. Insoluble phosphates (IPs) were extracted and eluted sequentially by ion exchange chromatography on Dowex columns according to methods published previously (6).

Src Kinase Activity Assays and Immunoprecipitation for EGFR and ErbB2—Src kinase activity was assayed on lysates from cells extracted for 10 min on ice in extraction buffer (50 mM Heps, pH 7.4, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM sodium deoxycholate, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM/µl leupeptin, 10 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride). c-Src was immunoprecipitated with 550 µg of lysates (precleared with protein G-Sepharose). Immunoprecipitates were successively washed with extraction buffer, buffer A (20 mM Tris, pH 7.4, 0.5 mM LiCl, 1 mM EDTA), and buffer B (20 mM Tris, pH 7.4, 10 mM MnCl, 1 mM EGTA) followed by incubation with 30 µg kinase buffer containing 20 µM Tris, pH 7.4, 10 mM MnCl, 1 mM EDTA, 5 mM MgCl2, 5 µM cold ATP, and 10 µCi of [32P]ATP with 1 mg/ml enolase as substrate for 15 min at 20 °C. The reactions were stopped with sample buffer, and the proteins were separated by SDS-PAGE (10% gel); quantification was with a PhosphorImager (Molecular Dynamics). The precleared lysates (700 µg) were also subjected to immunoprecipitation using anti-EGFR and anti-ErbB2 antibodies (Santa Cruz Biotechnology) followed by immunoblotting with anti-phosphotyrosine, anti-EGFR and anti-ErbB2 according to the manufacturer’s instructions.

Immunoblot Analysis for ERK and p38-MAPK Activation—Activation of ERK and p38-MAPK was monitored by immunoblot analysis with antibodies for phosphorylated (activated) signaling proteins. Where indicated, PPI or AG1478 (Calbiochem) were included (starting 45 min prior to the stimulatory intervals) to inhibit the kinase activities of Src family kinases or EGFR kinases, respectively. In all of the experiments, the immunoblots were stripped and reprobed with antibodies to total protein to confirm equal protein loading. Phospho-ERK1/2, total p38-MAPK, and phospho-p38-MAPK antibodies were from Cell Signaling Technology; antibodies to total ERK1/2 were from Santa Cruz Biotechnology. Immunoblotting was according to methods published previously or to the manufacturer’s instructions (12). Each panel in each figure represents results from a single gel exposed for a uniform duration, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry.

RESULTS

Reverse Transcraptase-PCR Analysis of PAR-4—Total RNA was isolated from embryonic mouse ventricular myocytes (embryonic day 18), cultured neonatal rat ventricular myocytes (postnatal day 2), and ventricular tissue from adult mouse and rat hearts converted to first strand cDNA with reverse transcriptase and used as templates in analytical PCR reactions. Total RNA from rat platelets was included as a positive control. The reaction products were analyzed by Southern blot hybridized with appropriate nested primer probes. Fig. 1 (upper panel) shows that a band corresponding in size to that predicted by the sequence of the primer pairs for PAR-4 is detected in all of the samples. The reaction product is prominent in platelets, but it also is readily detected in samples from adult rodent ventricles and (at lower levels) in neonatal rat and mouse cardiomyocyte cultures. In cardiomyocyte preparations, a band that migrates more rapidly (and likely corresponds to a smaller nested product from internal priming) also is consistently detected. Equivalent levels of reaction product for β-actin verify uniform loading, and β-actin is detected only in reactions that include reverse transcriptase performed in parallel as negative controls. These studies identify PAR-4 transcripts in mouse and rat cardiomyocytes.

PAR-4 Signaling Pathways in Cardiomyocytes—Consistent with previous studies establishing functional PAR-1 expression in mouse cardiomyocyte cultures (10), Fig. 2 shows that SFLRN induces a rapid/brief increase in inositol polyphosphates (IP2/IP3) that is followed by a more progressive and
sustained accumulation of IP$_1$. Although SFLLRN has known agonist activity at both PAR-1 and PAR-2 (13), the PAR-2-selective agonist peptide SLIGRL does not promote IP accumulation in mouse cardiomyocytes cultures (data not shown), excluding a significant contribution of PAR-2 to the actions of SFLLRN. To determine whether mouse cardiomyocytes express functional PAR-4, the cultures were stimulated with AYPGKF, a PAR-4-specific agonist peptide that is reported to be ~10-fold more potent than the PAR-4 tethered ligand sequence GYPGKF at activating PAR-4 (14). Fig. 2 shows that AYPGKF promotes phosphoinositide hydrolysis in mouse cardiomyocytes. However, the characteristics of signaling by AYPGKF-activated PAR-4 and SFLLRN-activated PAR-1 differ in two major respects: 1) the magnitude of IP accumulation (both IP$_2$/IP$_3$ and IP$_1$) in response to AYPGKF is relatively modest, compared with the robust responses elicited by SFLLRN and 2) the kinetics of IP$_2$/IP$_3$ accumulation in response to AYPGKF is atypically delayed. SFLLRN-induced IP$_2$/IP$_3$ accumulation is detected maximally at early time points (2–5 min); this response wanes at 30 min. In contrast, little AYPGKF-dependent IP$_2$/IP$_3$ accumulation is detected at 5 min; a modest AYPGKF-dependent increase in IP$_2$/IP$_3$ accumulation is observed at 30 min; however, long-term incubation with AYPGKF results in a sustained increase in IP accumulation. AYPGKF-dependent activation of phospholipase C is PTX-insensitive (data not shown).

Fig. 3 compares AYPGKF and SFLLRN activation of MAPK cascades in cardiomyocytes. AYPGKF activates ERK, but the magnitude of this response is modest and the kinetics are protracted relative to the brisk ERK activation induced by thrombin and SFLLRN. The weak and sluggish nature of PAR-4 signaling to ERK parallels the protracted time course for PAR-4 agonist activation of phospholipase C. AYPGKF also activates p38-MAPK. Here, AYPGKF activation is substantial; it is to a level comparable with p38-MAPK activation by PAR-1 agonists (or stimuli such as sorbitol, data not shown). However, the kinetics of p38-MAPK activation by SFLLRN and AYPGKF differ. p38-MAPK activation by SFLLRN (or thrombin) peaks at 5 min and is sustained for at least 30 min. In contrast, the onset of p38-MAPK activation by AYPGKF is delayed, with little increase in p38-MAPK activity in response to AYPGKF during incubations shorter than 20 min. Previous studies identified the effects of thrombin and SFLLRN to promote cardiomyocyte hypertrophy, as manifest by a significant increase in [$^3$H]phenylalanine incorporation into protein as well as total protein content (10). AYPGKF also is a weak hypertrophic agonist, inducing a 19.3 ± 2.3% increase in [$^3$H]phenylalanine incorporation into protein (compared with a 43.7 ± 3.2% increase by thrombin, n = 3 for each, p < 0.05 versus basal).

Src has been implicated in many of the cellular actions of thrombin. To determine whether Src contributes to cardiomyocyte activation by thrombin, the cardiomyocytes were exposed to vehicle or PAR agonists, and the intrinsic kinase activity of Src was determined by an immune complex kinase assay. Fig. 4A shows that thrombin increases the kinase activity of Src toward the exogenous substrate enolase. In contrast, Src is not activated by SFLLRN. This result is surprising, because thrombin-dependent activation of Src generally is ascribed to a signaling pathway emanating from PAR-1 (which would be activated by SFLLRN). This pharmacologic profile suggested that Src might lie downstream from PAR-1 (not PAR-1) in cardiomyocytes. Fig. 4A shows that AYPGKF induces a modest level of Src activation, consistent with this formulation.

The PAR-1 requirement for thrombin-dependent activation of Src was explored further in cardiomyocytes cultured from PAR-1$^{-/-}$ mice. This model was first validated by demonstrating that SFLLRN and the purinergic agonist ATP activate phospholipase C and ERK in wild type cardiomyocytes, but only ATP activates phospholipase C and ERK in PAR-1$^{-/-}$ cardiomyocytes (Fig. 5, A and B). Although PAR-1$^{-/-}$ cardiomyocytes are unresponsive to SFLLRN, effects of thrombin to activate p38-MAPK and Src persist in PAR-1$^{-/-}$ cardiomyocytes (Figs. 4, B and C, and 5C); AYPGKF-dependent activation of p38-MAPK and Src also is detected in this preparation. Collectively, these results indicate that cardiomyocyte responses to thrombin are mediated by the combined actions of PAR-1 and PAR-4. PAR-1 activates the phospholipase C/ERK pathway in cardiomyocytes.
pathway and also provides a mechanism to activate p38-MAPK. In contrast, PAR-4 promotes only a low level of sustained phospholipase C/ERK activation; its more prominent action is to stimulate p38-MAPK and Src. Surprisingly, thrombin-dependent activation of Src is mediated only by PAR-4 in cardiomyocytes (and not PAR-1).

To determine whether Src lies upstream from p38-MAPK in the PAR-4 signaling pathway, further studies were performed with PP1 (a specific inhibitor of Src family tyrosine kinases). Fig. 6 shows that PP1 blocks p38-MAPK and ERK activation by AYPGKF; this is not due to a nonspecific inhibitory effect of PP1, because ERK and p38-MAPK activation by sorbitol is equivalent in control and PP1-treated cultures. Given the recent evidence that hypertrophic signaling by cardiomyocyte G protein-coupled receptors also can involve EGFR transactivation (17), AYPGKF responses also were examined in cultures pretreated with AG1478 (a well established inhibitor of the kinase activity of the EGFR (18, 19)). Fig. 6 shows that AG1478 also inhibits ERK and p38-MAPK activation by AYPGKF (but not sorbitol) in cardiomyocytes. Separate experiments demonstrated that PAR-4 activation of p38-MAPK and Src is not blocked by PTX (data not shown). Collectively, these results implicate Src and EGFR kinases activities, but not G proteins, in the pathway for PAR-4-dependent activation of ERK and p38-MAPK in cardiomyocytes.

PAR-4 Signaling in Cells That Stably Overexpress PAR-4—PAR-4 expression reconstitutes thrombin-dependent activation of ERK and p38-MAPK in fibroblasts from PAR-1−/− mice (Fig. 7), providing a model system to resolve the mechanism(s) underlying the actions of PAR-4. The goal of studies in PAR-4 expressing cells was to determine whether separate signaling pathways for PAR-4 could be resolved (and whether PAR-4 signaling in cardiomyocytes conforms to a defined subset of the actions of PAR-4). GF190203X was used to inhibit phorbol ester-sensitive protein kinase C isoforms and thereby ablate the phospholipase C-protein kinase C pathway. PP1 and AG1478 were used to inhibit the kinase activities of Src and EGFRs, respectively. The EGFR antagonist was included in these experiments as a control for the specificity of inhibition by PP1; it also provided a strategy to consider a potential role for EGFR transactivation in PAR-4 signaling.

Fig. 7 shows that thrombin induces a robust increase in ERK and p38-MAPK activity at 5 min; ERK activation wanes slightly at 30 min, whereas p38-MAPK activation persists dur-

FIG. 4. Src activation by thrombin and AYPGKF in cardiomyocytes from wild type and PAR-1−/− mice. Serum-starved cardiomyocytes were exposed to vehicle, thrombin (1 unit/ml), SFLLRN (300 μM), or AYPGKF (500 μM) for 10 min. Src activity with enolase as substrate was measured as described under “Materials and Methods.” Representative experiments are illustrated in A and B, with results obtained in three separate culture preparations quantified in C. WT, wild type.

FIG. 5. SFLLRN activation of phospholipase C and ERK is lost, but thrombin and AYPGKF activation of p38-MAPK persists, in PAR-1−/− cardiomyocytes. Cardiomyocyte cultures from outbred mice were stimulated with vehicle, SFLLRN (300 μM), or ATP (100 μM) for 30 min (A) or 5 min (B). A, inositol phosphate accumulation was determined by Dowex column chromatography, and ERK activation was identified by SDS-PAGE and Western blotting of cell lysates with anti-phospho-ERK1/2 antibody. The control experiments established that ERK protein expression is equivalent in wild type and PAR-1−/− cultures. B, incubations were with AYPGKF (500 μM) or thrombin (1 or 10 units/ml) for the indicated intervals, with p38-MAPK activation tracked with an antibody specific for the phosphorylated (activated) species. The results are the means ± S.E. from triplicate determination in three separate cultures (A) or representative autoradiograms (with each lane from a single gel exposed for the same duration) from a single experiment, with equivalent results in two other culture preparations, in B and C. WT, wild type; P, phospho.

FIG. 6. PAR-4-dependent stimulation of ERK and p38-MAPK requires intact Src and EGFR kinase activity in cardiomyocytes. Serum-starved cardiomyocyte cultures from outbred mice were treated with vehicle, AYPGKF (500 μM), or sorbitol (0.5 M) for 30 min without or with PP1 (10 μM) or AG1478 (2 μM; Calbiochem) pretreatment, starting 45 min prior to stimulation. The efficacy of the AG1478 pretreatment protocol was established in separate experiments demonstrating complete inhibition of ERK and AKT activation by heregulin (data not shown). Western blotting was with the anti-phospho-p38-MAPK or anti-phospho-ERK antibodies; immunoblot analysis with total ERK1/2 and p38-MAPK established constant protein loading in all lanes (data not shown). Similar results were obtained in three separate experiments. P, phospho.
ing this interval. PP1 is a generalized inhibitor of thrombin-dependent activation of ERK and p38-MAPK at both early and late time points. This result implicates Src kinases in all aspects of PAR-4 signaling. At the early time point, thrombin-dependent activation of ERK also is blocked by GF109203X; GF109203X does not inhibit ERK activation at the later time point or thrombin-dependent activation of p38-MAPK at either time point. The PAR-4 signaling phenotype in GF109203X-treated cells is reminiscent of PAR-4 signaling in cardiomyocytes, where AYPGKF induces prominent p38-MAPK activation and ERK activation with delayed kinetics.

The more delayed component of thrombin-dependent activation of ERK and p38-MAPK is blocked by the EGFR antagonist AG1478 or PP1 (and not by GF109203X). Thrombin-dependent activation of ERK at 30 min is inhibited by PP1 and AG1478, whereas EGF-dependent activation of ERK is blocked only by AG1478 (not by PP1). This result establishes the efficacy of inhibition by AG1478, as well as the specificity of PP1 for Src (and not the EGFR). This result also places Src upstream from EGFRs in the PAR-4 signaling pathway leading to ERK activation (although further evidence that PP1 effectively blocks EGF-dependent activation of p38-MAPK indicates that Src also exerts a role downstream from EGFRs in the pathway leading to p38-MAPK activation).

The pharmacologic studies implicate EGFR transactivation in the PAR-4 signaling pathway. These results are quite novel; there is precedent for PAR-1 transactivation of EGFR and the related EGFR family member ErbB2 (20), but EGFR transactivation as a mechanism for signaling by PAR-4 has never been reported. Because preliminary studies indicated that PAR-4-expressing fibroblasts from PAR-1<sup>−/−</sup> mice express EGFRs and ErbB2 (data not shown), we explored the potential individual roles for EGFRs and ErbB2 in the PAR-4-dependent signaling pathway. Fig. 8 shows that thrombin promotes a large increase in the tyrosine phosphorylation of ~170–175- and ~180–185-kDa proteins, respectively, in anti-EGFR and anti-ErbB2 immunoprecipitates from PAR-4 expressing PAR-1<sup>−/−</sup> fibroblasts; thrombin-dependent tyrosine phosphorylation is largely blocked by the Src kinase inhibitor PP1 (but not by PP3, a structurally analogous compound that does not inhibit Src family kinases, attesting to the specificity of the inhibitory actions of PP1; data not shown). The direct activation/phosphorylation of EGFR (by EGF) and ErbB2 (by EGF and heregulin) is not blocked by PP1; to the contrary, EGFR-dependent tyrosine phosphorylation of ErbB2 is slightly increased, and heregulin-induced tyrosine phosphorylation of ErbB2 is markedly in-
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We thank Ema Stasko for preparing myocyte cultures.

creased in PP1-treated cultures. A similar effect of PP1 to increase EGF phosphorylation of ErbB2 was identified previously (21). The blot was stripped and reprobed with anti-EGFR and ErbB2 to validate equal protein loading. EGFR immunoreactivity is detected at grossly similar levels in all of the samples. ErbB2 also is detected at similar levels in samples from unstimulated, thrombin-stimulated, and heregulin-stimulated cultures, but ErbB2 protein appears to be reduced in EGF-stimulated samples (and in the EGF- and heregulin-stimulated samples from PP1-treated cultures). However, this is an artifact, related to the sequence for immunoblotting in this experiment, because ErbB2 is detected at similar levels in all samples when the immunoblot is first probed with anti-ErbB2 antibody (data not shown). This suggests that extensive signals with the anti-phosphotyrosine antibody prevent subsequent anti-ErbB2 antibody binding to the ErbB2 protein epitope.

Collectively, these studies effectively resolve two parallel Src-dependent signaling pathways emanating from PAR-4 (as schematized in Fig. 9). The initial rapid PAR-4-dependent activation of ERK involves Gq/phospholipase C-dependent activation of protein kinase C and Src. The more sustained PAR-4-dependent activation of ERK (and p38-MAPK) is via a separate Src-dependent pathway that involves EGFR and/or ErbB2 transactivation. This latter Src-p38-MAPK pathway dominates in cardiomyocytes.

**DISCUSSION**

These studies provide novel evidence that cardiomyocytes express functional PAR-4 and that the signaling properties of PAR-4 in cardiomyocytes are distinct from those previously reported for PAR-1. Specifically, PAR-1 promotes the rapid/strong activation of phospholipase C and ERK (via PTX-sensitive and -insensitive pathways) and a more sustained activation of p38-MAPK. Although human PAR-4 (expressed in PAR-1−/− fibroblasts) supports thrombin-dependent activation of all of these responses (phospholipase C, ERK, and p38-MAPK), PAR-4 native to mouse cardiomyocytes preferentially elicits a subset of these responses. PAR-4 stimulation leads to a relatively strong activation of p38-MAPK, with only a minor associated increase in phospholipase C and ERK that is delayed in onset. Additional studies implicate tyrosine phosphorylation events on both EGFRs and ErbB2 in the actions of PAR-4, providing the first evidence that transactivation of EGFR family members can contribute to PAR-4 responses. The protracted kinetics for PAR-4 signaling in cardiomyocytes presumably is attributable to the slow activation and desensitization kinetics described for PAR-4 (relative to PAR-1 (5)). However, a mechanism that accounts for the very weak PAR-4 coupling to the phospholipase C/ERK pathway is not obvious. It is tempting to speculate that this lesion in PAR-4 signaling in mouse cardiomyocytes is part of a more generalized defect in GqPCR-dephospholipase C/ERK pathway is not obvious. It is tempting to

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The predominant natural activator(s) of endogenous car-
diomyocyte PARs remains uncertain. Although cardiomyocyte PAR-1 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. Hence, other potential mechanisms for PAR activation must be considered. PAR-4 has been identified as a potential substrate for neutrophil-derived proteases that are released at sites of cardiac injury and/or inflammation (26). Hence, the cardiac actions of PAR-4 may become important in the context of cardiac remodeling during myocarditis and/or at the border zone of a myocardial infarction.

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