Neutrophil Cathepsin G Promotes Detachment-induced Cardiomyocyte Apoptosis via a Protease-activated Receptor-independent Mechanism*

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Cathepsin G is a neutrophil-derived serine protease that contributes to tissue damage at sites of inflammation. The actions of cathepsin G are reported to be mediated by protease-activated receptor (PAR)-4 (a thrombin receptor) in human platelets. This study provides the first evidence that cathepsin G promotes inositol 1,4,5-trisphosphate accumulation, activates ERK, p38 MAPK, and AKT, and decreases contractile function in cardiomyocytes. Because some cathepsin G responses mimic cardiomyocyte activation by thrombin, a role for PARs was considered. Cathepsin G markedly activates phospholipase C and p38 MAPK in cardiomyocytes from PAR-1−/− mice, but it fails to activate phospholipase C, ERK, p38 MAPK, or AKT in PAR-1- or PAR-4-expressing fibroblasts (which display robust responses to thrombin). These results argue that PAR-1 does not mediate the actions of cathepsin G in cardiomyocytes, and neither PAR-1 nor PAR-4 mediates the actions of cathepsin G in fibroblasts. Of note, prolonged incubation of cardiomyocytes with cathepsin G results in the activation of caspase-3, cleavage of FAK and AKT, sarcomeric disassembly, cell rounding, cell detachment from underlying matrix, and morphologic features of apoptosis. Inhibition of Src family kinases or caspsases (with PP1 or benzoyloxy carbonyl-Val-Ado-fluoromethyl ketone, respectively) delays FAK and AKT cleavage and cardiomyocyte detachment from substrate. Collectively, these studies describe novel cardiac actions of cathepsin G that do not require PARs and are predicted to assume functional importance at sites of interstitial inflammation in the heart.

Cathepsin G is a major serine protease released by activated neutrophils at sites of vascular injury and inflammation (1, 2). Cathepsin G is a strong agonist for platelets, causing a rise in intracellular calcium, aggregation, and degranulation. Cathepsin G induces functional and morphological changes in endothelial cells that lead to increased permeability and growth factor release (1, 3–5). Cathepsin G also induces tissue injury and contributes to the pathogenesis of certain chronic pulmonary diseases, such as emphysema and cystic fibrosis (6). These diverse cellular actions of cathepsin G have been attributed to the cleavage and function modulation of a range of protein substrates, including clotting factors (factor V and factor VII), neutrophil chemoattractants (tumor necrosis factor α, interleukin-1, and interleukin-8), and matrix components (including collagen, fibronectin, and elastin). Recent studies have focused on cathepsin G-dependent cleavage of seven transmembrane spanning domain G protein-coupled protease-activated receptors (PARs) as an additional potentially important mechanism whereby cathepsin G modulates coagulation and tissue remodeling at sites of injury and inflammation. PAR-1, the prototypical receptor for thrombin, is activated as a result of thrombin-dependent cleavage of its extracellular N terminus to expose a new N-terminal sequence (SFLLRN) that binds intramolecularly and serves as a tethered ligand (7). Since the initial cloning of PAR-1, three additional structurally homologous PARs have been identified; PAR-3 and PAR-4 are activated by thrombin, whereas PAR-2 is activated by trypsin, membrane-type serine protease-1, and mast cell tryptase (but not thrombin) (8, 9). PARs are endowed with two unique regulatory features as a result of their distinctive proteolytic activation mechanism. PAR-1, PAR-3, and PAR-4 need not be entirely selective for thrombin but are predicted to be susceptible to activation by any serine protease capable of cleaving the N terminus at the site that exposes the tethered ligand sequence. Additionally, PAR cleavage at sites on the N terminus more proximal to the transmembrane domain is predicted to amputate the tethered ligand sequence and render the PAR unresponsive to subsequent proteolytic activation. Cathepsin G is reported to modulate PAR function via both mechanisms. At concentrations achieved in the vicinity of activated neutrophils, cathepsin G mimics the action of thrombin to mobilize intracellular calcium in Xenopus oocytes and fibroblast cell lines that stably overexpress human PAR-1 or human PAR-4 (but not in untransfected cells or cells that overexpress PAR-2 or PAR-3). Mutagenesis studies suggest that in each case the mechanism entails cleavage of the N terminus (at the Arg45–Ser46 bond in PAR-1 and the Arg87–Gly88 bond in PAR-4) to expose the tethered ligand (1, 10). These results suggest that.

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† The abbreviations used are: PARs, protease-activated receptors; IP, inositol phosphate; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; Z, benzoyloxy carbonyl; fmk, fluoromethyl ketone; PTX, pertussis toxin; DMEM, Dulbecco’s modified Eagle’s medium; MMPs, matrix-degrading metalloproteinases; FAK, focal adhesion kinase.
The cardiovascular signaling properties of PARs generally have been explored in platelets (where the actions of thrombin are critical for normal hemostasis and arterial thrombosis) and that certain cathepsin G actions via distinct mechanisms) and that certain cathepsin G actions on PARs may be species-dependent.

Cardiac Actions of Cathepsin G

Cardiomyocytes—Cardiac myocytes were isolated from ventricles of Wistar rats (postnatal day two) or PAR-1 deficient mice (20, 21), which lack functional PAR-1, PAR-2, and PAR-4, were transfected with human PAR-1 or PAR-4 in a modified pCDNA3 construct encoding a hygromycin resistance gene. Stable transfectants were selected in 250 mg/ml of hygromycin B and screened by specific agonist-induced calcium mobilization. Cultures were maintained in DMEM containing 10% calf serum and 250 μg/ml hygromycin; for assays of MAPK or Akt activation, cultures were serum-starved overnight.

Measurement of Cytosolic Free Calcium and Cell Shortening—Intracellular calcium and shortening was simultaneously measured photometrically in fura-2 loaded cultured neonatal rat ventricular myocytes according to methods published previously (14). In brief, myocytes cultured on glass cover slips were washed three times with ice-cold PBS, free of calcium buffered saline (pH 7.4), scraped into hot SDS-PAGE sample buffer, sonicated, and then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was diluted in SDS-PAGE sample buffer, boiled for 5 min, and stored at −70 °C. Western blot analysis was performed according to manufacturer’s instructions, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were purchased commercially from the following sources: thrombin (Calbiochem); fura 2-AM (Molecular Probes); [3H]Hyaluronic Acid (34.4 Ci/mmol, PerkinElmer Life Sciences); pertussis toxin (PTX, List Biologicals). Cathepsin G was obtained from Calbiochem for most experiments; selected experiments were performed with cathepsin G from Sigma and yielded equivalent results. All other chemicals were reagent-grade and obtained from standard chemical suppliers. SFLLRN and AYPGKF were synthesized at the State University of New York, Stony Brook, as C-terminal amides, purified by high pressure liquid chromatography, and characterized by mass spectrometry. Peptide solutions were made fresh from powders for all experiments.

Cardiac myocytes were dissociated from the ventricles of Wistar rats (postnatal day two) or PAR-1−/− or background strain C57BL/6 mice (embryonic day 18–20) by a trypsin digestion protocol which incorporates a differential attachment procedure to enrich for cardiac myocytes (14). Although the preplating step effectively decreases fibroblast contamination, myocytes were subjected to 30 gray of X-rays on day 1 of culture to halt the proliferative potential of any residual contaminating fibroblasts (14). Cardiomyocytes were plated at a density of 0.5 × 10⁶ cells per ml (2 ml per 35-mm dish) and were cultured in DMEM supplemented with 10% fetal calf serum. For assays of ERK, p38 MAPK, or Akt activation, cells were serum-starved in 1:1 DMEM/F12 medium for 24 h.

Cell Lines—Immortalized murine lung myofibroblasts derived from PAR-1-deficient mice (20, 21), which lack functional PAR-1, PAR-2, and PAR-4, were transfected with human PAR-1 or PAR-4 in a modified pCDNA3 construct encoding a hygromycin resistance gene. Stable transfectants were selected in 250 mg/ml of hygromycin B and screened by specific agonist-induced calcium mobilization. Cultures were maintained in DMEM containing 10% calf serum and 250 μg/ml hygromycin; for assays of MAPK or Akt activation, cultures were serum-starved overnight.

Phosphoinositide Hydrolysis—Cells were incubated for 72 (cardiomyocytes) or 24 h (cell lines) with 3 μCi/ml [3H]Hyaluronic acid, washed with preincubuted with 10 mM LiCl for 20 min, and then stimulated with agonists for the indicated intervals at room temperature. Inositol phosphates (IPs) were extracted and eluted sequentially by ion-exchange chromatography on Dowex columns according to methods published previously (14).

Immunoblotting—Immunoblot analysis was used to compare levels of total and phosphorylated ERK, p38 MAPK, and Akt and to track the accumulation of caspase-3 and FAK cleavage products. Following exposure to test agents as indicated in individual experimental protocols, cells were washed three times with ice-cold PBS, free of calcium buffered saline (pH 7.4), scraped into hot SDS-PAGE sample buffer, sonicated, and then centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was diluted in SDS-PAGE sample buffer, boiled for 5 min, and stored at −70 °C. Western blot analysis was performed according to manufacturer’s instructions, with bands detected by enhanced chemiluminescence and quantified by laser scanning densitometry. For each panel in each figure, the results are from a single gel exposed for a uniform duration.

Measurement of Cytosolic Free Calcium and Cell Shortening—Intracellular calcium and shortening was simultaneously measured photometrically in fura-2 loaded cultured neonatal rat ventricular myocytes according to methods published previously (14). In brief, myocytes cultured on glass cover slips were washed with fura-2 by incubation in Tyrode’s solution containing 3 μM of the acetoxymethyl ester form of fura-2 (fura-2/AM) and 1.5 μM of 25% (w/v) in dimethyl sulfoxide) Pluronic F-127 for 20 min at 37 °C. Cardiomyocytes were rinsed with fresh Tyrode’s solution and maintained for at least 15 min at room temperature to allow for de-esterification of the dye. Cardiomyocytes were superfused with room temperature Tyrode’s solution gassed with 95% O₂, 5% CO₂ at a rate of 1 ml/min and paced by electrical field stimulation at 1 Hz throughout the experimental protocol (to avoid changes in cell calcium due to potential chronotropic actions of agonists). Drugs were introduced as a bolus into the prechamber of a three compartment superfusion chamber described previously (14). Intracellular fura-2 fluorescence was monitored (at a sampling rate of 100 Hz) with a device that alternately illuminates the cells with 340 and 380 nm light while measuring emission at 505 nm. Fluorescence images were captured and then stacked by Spot software, and monochrome images were calibrated to convert to micrometers of movement. The motion preparation. Therefore, relative cell motion rather than actual cell length is reported. The analog voltage output from the motion detector was calibrated to convert to micrometers of movement. The motion signal, obtained at a rate of 60 Hz, was digitized and stored using Deltascan software (Photon Technology Int.).

Time-lapse Video and Fluorescence Microscopy—Cells were grown on 25-mm slides that were mounted in sealed rose chambers maintained at 37 °C, in the presence of media with or without cathepsin G. Images were taken every 5 min for ~24 h with a Spot RT Monochrome CCD camera (Diagnostics Instruments) on a Nikon Eclipse TE200 Phase contrast microscope equipped with differential interference contrast microscope. Some slides were stained with Hoechst 33342 or 33258 and imaged through a UV-2A filter cube. The Uniblitz Shutter (model VMM01) was controlled by Spot RT software or Scion Imaging (National Institutes of Health). Pictures were captured and then stacked by Spot software, and montages were prepared through Photoshop software.
RESULTS

Cathepsin G Promotes Phosphoinositide Hydrolysis and Activates ERK, p38 MAPK, and AKT in Cardiomyocytes; Distinct Signaling by Cathepsin G and PAR Agonists—Fig. 1 shows that cathepsin G markedly increases IP accumulation in neonatal rat cardiomyocytes. Responses are detected at relatively low concentrations of cathepsin G, typical of those reported to be generated at sites of inflammation. The characteristics of IP accumulation in response to conventional PAR-1 agonists (thrombin, SFLLRN) versus cathepsin G are quite distinct. Consistent with previous results, PAR-1 activation by maximally stimulatory concentrations of thrombin (1 unit/ml) or the PAR-1 agonist peptide SFLLRN (300 µM) results in transient elevations of IP$_2$/IP$_3$, followed by a more sustained accumulation of IP$_1$ (22). In contrast, cathepsin G promotes the progressive accumulation of IP$_2$ and IP$_3$ (to levels as high or higher than the levels transiently observed in cells stimulated with thrombin or SFLLRN); the accompanying cathepsin G-induced rise in IP$_1$ levels is trivial in comparison. Because we recently identified PAR-4 as an additional cellular receptor for thrombin in cardiomyocytes (19), we also compared responses to cathepsin G and AYPGKF (a derivative of the PAR-4 tethered ligand sequence that is highly specific for PAR-4 and acts at ~10-fold lower concentrations than the tethered ligand sequence GYPGKF itself) (23). Table I shows that AYPGKF induces a rise in IP$_1$ levels, but the magnitude of this response is relatively modest compared with the rise in IP$_1$ levels elicited by 1 unit/ml thrombin (a concentration that maximally activates phosphoinositide hydrolysis in cardiomyocytes). AYPGKF actions cannot be attributed to robust PAR-4 signaling in a minor contaminating fibroblast population, because cardiac fibroblasts display brisk/pronounced increases in IP accumulation in response to thrombin and SFLLRN, but cardiac fibroblasts do not respond to AYPGKF (24). Of note, stimulation with AYPGKF results in the sustained accumulation of

![Fig. 1. Cathepsin G promotes $[^{3}$H$]IP accumulation in cultured neonatal rat ventricular myocytes. $[^{3}$H$]Inositol-labeled rat ventricular myocytes cultures were incubated with SFLLRN (300 µM), thrombin (1 unit/ml), or cathepsin G (400 nM) for the indicated intervals (A), or with increasing concentrations of cathepsin G for 30 min (B, note 0.02 units/ml corresponds to 400 nM cathepsin G); IP metabolites were extracted and separated by Dowex anion-exchange chromatography as described under “Experimental Procedures.” Results are expressed as counts/min over the corresponding controls for triplicate determinations from three experiments (mean ± S.E.). Similar results were obtained in separate experiments on cardiac cultures prepared from mouse ventricles (data not shown).](https://example.com/fig1.png)

![TABLE I

|                     | IP$_1$  | IP$_2$ + IP$_3$ |
|---------------------|---------|-----------------|
| Basal               | 475 ± 48| 95 ± 6          |
| Thrombin            | 1632 ± 65*| 144 ± 18*      |
| AYPGKF              | 648 ± 50*| 126 ± 12*       |
| Cathepsin G         | 542 ± 74| 327 ± 22*       |

* $p < 0.05$ versus basal.
IP\(_3\), with only a relatively minor associated increase in IP\(_2\)/IP\(_3\) levels. This is quite distinct from the effect of cathepsin G, which promotes the preferential accumulation of IP\(_2\)/IP\(_3\). The actions of cathepsin G and thrombin are distinguished further based upon their sensitivities to the inhibitory effects of PTX. Previous studies established that the effect of thrombin to activate phospholipase C in cardiomyocyte cultures is severely curtailed by pretreatment with PTX (22). In contrast, IP\(_2\)/IP\(_3\) accumulation in response to cathepsin G is PTX-insensitive (IP\(_2\) + IP\(_3\) accumulation (CPM over basal): control 238 ± 14; PTX 253 ± 22, \(n = 3\); not significant). Collectively, these experiments identify rather distinct IP responses to cathepsin G versus conventional PAR agonists in cardiomyocytes, suggesting that PARs are unlikely mediators of the cardiac actions of cathepsin G.

The studies next examined whether cathepsin G activates ERK, p38 MAPK, and AKT, effectors previously identified as targets of PARs in cardiomyocytes. Fig. 2 shows that cathepsin G activates ERK, p38 MAPK, and AKT in cardiomyocyte cultures. Incubations were with 400 nM cathepsin G for the indicated time intervals or with 1 unit/ml thrombin (Thr) for 5 min. Cell lysates were subjected to SDS-PAGE and Western blotting with specific anti-phospho-ERK1/2 (A), −p38 MAPK (B), or −AKT (C) antibodies; stripped blots were subsequently analyzed for total ERK1/2, p38 MAPK, or AKT protein. Top, representative autoradiograms (with each lane from a single gel exposed for the same duration). Bottom, quantification of experiments from three separate cultures.

**Fig. 2.** Cathepsin G activates ERK, p38 MAPK, and AKT in cardiomyocyte cultures. Incubations were with 400 nM cathepsin G for the indicated time intervals or with 1 unit/ml thrombin (Thr) for 5 min. Cell lysates were subjected to SDS-PAGE and Western blotting with specific anti-phospho-ERK1/2 (A), −p38 MAPK (B), or −AKT (C) antibodies; stripped blots were subsequently analyzed for total ERK1/2, p38 MAPK, or AKT protein. Top, representative autoradiograms (with each lane from a single gel exposed for the same duration). Bottom, quantification of experiments from three separate cultures.
G promotes a rapid and transient increase in ERK1/2 phosphorylation; the response is maximal at 5 min and wanes thereafter. The magnitude of cathepsin G-dependent activation of ERK is relatively modest, compared with the robust response induced by thrombin. In contrast, cathepsin G markedly activates p38 MAPK and AKT (to levels equivalent to or slightly exceeding the response to thrombin) but with relatively slow kinetics. Activation of p38 MAPK and AKT is detectable at 5 min and increases progressively during the first 30 min of stimulation. These results suggest that cathepsin G and PARs activate certain common target effectors. However, differences in the kinetics and the magnitude of these responses suggest distinct activation mechanisms.

Fig. 3 shows that cathepsin G also depresses the amplitude of cell shortening (43.9 ± 9.6%, n = 11, p < 0.01). The PAR-1 agonist SFLLRN induces a similar decrease in contractile function (61.4 ± 10.5%, n = 13, p < 0.01). However, the effects of cathepsin G and SFLLRN on intracellular calcium regulation are quite different. SFLLRN increases diastolic and peak systolic calcium ion concentration (30.5 ± 4.7 and 28.7 ± 8.8%, respectively, n = 13, p < 0.01), whereas cathepsin G does not significantly alter intracellular calcium. The effect of cathepsin G to depress contractile function, without altering intracellular calcium, is most consistent with a mechanism that reduces myofibrillar calcium sensitivity.

Stimulatory Actions of Cathepsin G Do Not Require PAR-1 or PAR-4—Certain aspects of the cellular response to cathepsin G (in particular the unusually slow/progressive accumulation of IP$_2$/IP$_3$, without an associated rise in IP$_1$) are sufficiently distinct from the response to traditional PAR agonists to suggest that cathepsin G might act via a distinct signaling mechanism. Therefore, the studies next took advantage of cardiomyocytes cultured from ventricles of wild-type and PAR-1$^{-/-}$ mice to investigate the role of PAR-1 in the stimulatory actions of cathepsin G. Fig. 4 shows that SFLLRN promotes IP accumulation and activates p38 MAPK in cardiomyocytes cultured from wild-type, but not PAR-1$^{-/-}$, mice. Control experiments establish that the defect in SFLLRN activation cannot be attributed to a lesion in components of the signaling pathway distal to PAR-1, because IP accumulation in response to purinergic receptor activation with ATP is robust in both wild-type and PAR-1$^{-/-}$ cultures (Fig. 4A). In contrast to SFLLRN responses, which require expression of a PAR-1 gene product, cathepsin G promotes IP accumulation and stimulates p38 MAPK in cardiomyocytes from both wild-type and PAR-1$^{-/-}$ mice. These results indicate that the stimulatory actions of cathepsin G in cardiomyocytes do not require PAR-1 expression.

Fibroblast cell lines derived from PAR-1$^{-/-}$ mice that stably express human PAR-1 or PAR-4 also were used to explore a possible PAR requirement for cathepsin G signaling. Fig. 5A shows that thrombin and PAR agonist peptides activate phosphoinositide hydrolysis, with the predicted differences in peptide agonist specificity and potency for thrombin. IP accumulation is maximally elicited by SFLLRN or 1 unit/ml thrombin in cells that overexpress PAR-1 and by AYPGKF or 10 units/ml thrombin in cells that overexpress PAR-4 (a receptor optimally cleaved by higher thrombin concentrations). In each case, receptor stimulation (proteolytically with thrombin or with peptide agonist) results in the activation of ERK, p38 MAPK, and AKT pathways (Fig. 5, B and C). In contrast, cathepsin G promotes only a very minor increase in the levels of IP$_1$ or IP$_2$/IP$_3$ in cells that overexpress PAR-4; cathepsin G does not detectably elevate IP levels above basal in cells that express PAR-1 (Fig. 5A). Although cathepsin G weakly activates ERK (but not p38 MAPK or AKT) in PAR-1- and PAR-4- expressing
cell lines (Fig. 5, B and C), this response displays atypically slow kinetics (peak at 10 min, relative to ERK activation by PARs which is maximal at 5 min). Cathepsin G also induces a similar weak and delayed activation of ERK in PAR−/− cells that do not respond to thrombin or peptide agonists (Fig. 5D). This indicates that the low level of ERK activation by cathepsin G cannot be ascribed to activation of either PAR-1 or PAR-4.

Cells that overexpress PAR-1 maintain a spread morphology and remain adherent to substrate during incubation with cathepsin G. In contrast, cells that overexpress PAR-4 round and detach from the underlying matrix during incubation with cathepsin G. These morphological changes do not result from PAR-4 activation, because intense PAR-4 stimulation with either thrombin or AYPGKF does not induce comparable morphologic changes. More likely, the very minor signaling responses induced by cathepsin G in cells that overexpress PAR-4 (in the context of cell detachment from matrix) represent the consequences of cathepsin G-induced alterations in cell adhesion and not proteolytic activation of PAR-4 by cathepsin G. Collectively, these results raise serious doubts that the agonist actions of cathepsin G can be attributed to the activation of either PAR-1 or PAR-4.

**Cathepsin G Disables PAR-1 but Not PAR-2 or PAR-4**—Cathepsin G cleavage could disable, rather than activate, PARs. Fig. 6A shows that preincubation with cathepsin G for 30 min completely abrogates subsequent IP3 accumulation induced by thrombin in cells that overexpress PAR-1; responses to SFLLRN remain intact, indicating that the receptor remains otherwise structurally intact. Whereas PAR-1 is highly susceptible to cathepsin G-dependent cleavage, preincubation with cathepsin G does not impair subsequent phosphoinositide hydrolysis induced by either thrombin or AYPGKF in cells that overexpress human PAR-4 (Fig. 6B). These results provide further evidence that PAR-4 is not a direct target for cathepsin G actions. Fig. 6C also shows that cathepsin G disables PAR-1 in rat cardiomyocytes; treatment with cathepsin G renders cardiomyocyte cultures refractory to subsequent stimulation by thrombin but not by SFLLRN. These results are noteworthy, because the disabling properties of cathepsin G were previously identified in the context of the human PAR-1 sequence. Because the putative cathepsin G inactivation site in human PAR-1 (Phe55–Trp56) is not conserved in the rat PAR-1 sequence, there must be additional sites for cathepsin G cleavage.

**Cathepsin G Induces Cardiomyocyte Detachment from Underlying Matrix and Apoptosis**—Cathepsin G is reported to alter endothelial cell morphology, leading to increased monolayer permeability and the generation of a potentially thrombogenic surface (25). These effects of cathepsin G are distinct from those induced by PAR-1 activation; cathepsin G does not detectably activate PAR-1 in endothelial cells. Cathepsin G induces similar changes in the morphology of cardiomyocytes. Fig. 7A shows phase contrast microscopy of an identical area of PAR−/− mice that stably express PAR-1 (A and B) or PAR-4 (A and C) or the parental line (D) were incubated with the indicated concentrations of cathepsin G, 300 μM SFLLRN, 500 μM AYPGKF, and thrombin (as indicated in A, 1 unit/ml in B, and 10 units/ml in C and D), or phorbol 12-myristate 13-acetate (PMA) (100 nM). A, IP metabolites that accumulate in the aqueous extracts of [3H]inositol-labeled cultures were isolated by Dowex anion-exchange chromatography as described under “Experimental Procedures.” Results are expressed as counts/min over the corresponding controls for triplicate determinations from a single experiment (mean ± S.E.). Similar results were obtained in two separate experiments. B, cell lysates were subjected to SDS-PAGE and Western blotting with anti-phospho-ERK, p38 MAPK, or AKT as described under “Experimental Procedures” (with subsequent immunoblot analysis of total ERK1/2, p38 MAPK, or AKT protein levels on stripped blots).

![Fig. 5. PAR-1 and PAR-4 expression fail to confer cathepsin G responsiveness in PAR−/− lung fibroblasts. Lung fibroblasts from PAR−/− mice that stably express PAR-1 (A and B) or PAR-4 (A and C) or the parental line (D) were incubated with the indicated concentrations of cathepsin G, 300 μM SFLLRN, 500 μM AYPGKF, and thrombin (as indicated in A, 1 unit/ml in B, and 10 units/ml in C and D), or phorbol 12-myristate 13-acetate (PMA) (100 nM). A, IP metabolites that accumulate in the aqueous extracts of [3H]inositol-labeled cultures were isolated by Dowex anion-exchange chromatography as described under “Experimental Procedures.” Results are expressed as counts/min over the corresponding controls for triplicate determinations from a single experiment (mean ± S.E.). Similar results were obtained in two separate experiments. B, cell lysates were subjected to SDS-PAGE and Western blotting with anti-phospho-ERK, p38 MAPK, or AKT as described under “Experimental Procedures” (with subsequent immunoblot analysis of total ERK1/2, p38 MAPK, or AKT protein levels on stripped blots).](image-url)
a cardiomyocyte monolayer culture before and at various time points during exposure to cathepsin G. The images demonstrate a flattened and spread appearance of cardiomyocytes under control conditions. Cathepsin G induces a progressive loss of cell-cell and cell-matrix contacts, such that gaps form between neighboring cells and the cells round and progressively detach from underlying matrix. Phase contrast microscopy and measurements of protein recovery show pronounced cell detachment at 3 h, with little to no matrix-adherent cellular material remaining in cultures treated with cathepsin G for 5 h (Fig. 7B). Of note, the early cathepsin G-induced changes in cardiomyocyte morphology (within the first 0.5–1 h) are largely reversible if the cathepsin G-containing medium is removed and replaced with fresh medium (data not shown).

The effects of cathepsin G to release cardiomyocytes from anchorage-dependent binding to extracellular matrix could lead to anoikis, a specialized form of detachment-induced apoptosis that results from inadequate or inappropriate cell-matrix interactions (26). Therefore, additional studies were performed to determine whether cardiomyocytes treated with cathepsin G display the hallmark features of apoptosis. Fig. 8 shows that within 2 h of cathepsin G treatment, cardiomyocytes begin to shrink and detach from the matrix. Immunostaining with sarcomeric α-actinin (the actin-binding protein that decorates Z-bands) reveals disruption of the highly organized sarcomeric banding pattern characteristic of control cardiomyocyte cultures. Myofibrillar disarray also is accompanied by shrinkage and fragmentation of nuclei (mean ± S.D. area of nuclei: control cells 285 ± 119 μm² versus cathepsin G-treated cells 70 ± 18 μm²; n = 10 for each). Images at 2–3 h of cathepsin G treatment reveal prominent blebbing of the cell membrane, with many nuclei staining with permeable Hoechst 33342. However, the cells remain viable as very few nuclei stain with membrane-impermeable Hoechst 33258; the cells exclude trypan blue, and (rather remarkably) many cells with this grossly abnormal appearance continue to beat spontaneous...
of more rapidly migrating FAK immunoreactivity (whose size corresponds to the truncated FAK fragment generated via caspase cleavage) accumulates under these conditions (27).

AKT is another potential substrate for caspase-3 during anoikis (28). Indeed, cathepsin G initially induces AKT phosphorylation/activation (Fig. 2), but more prolonged incubation with cathepsin G leads to a decrease in the level of full-length AKT protein. Structural studies predict that caspase cleavage would generate a PH-deficient 44-kDa kinase domain and an isolated 16-kDa PH domain of AKT (28, 29). However, smaller molecular weight degradation products did not detectably accumulate in cardiomyocytes exposed to cathepsin G. Similar results were obtained previously and ascribed to the successive in vivo cleavage of AKT by other proteases, which generate fragments that accumulate only transiently, at levels that are too low, or at sizes that are too small to be detected (28). Importantly, the cleavage of AKT and FAK does not result from the generalized indiscriminate proteolysis of cellular proteins as levels of ERK1/2 and p38 MAPK remain constant under these conditions (Fig. 9 and data not shown).

There is evidence that caspase activation during apoptosis is triggered by Src family kinases (30, 31). To determine whether Src kinases contribute to the cardiac actions of cathepsin G, studies were performed with PP1 (a Src family kinase inhibitor). Fig. 10 shows that PP1 completely prevents the early cathepsin G-dependent activation/phosphorylation of AKT; PP1 also attenuates the early effect of cathepsin G to activate p38 MAPK, stimulate ERK, and promote IP accumulation (although IP accumulation in response to cathepsin G remains significantly elevated in PP1-treated cultures). Fig. 9 shows that PP1 markedly inhibits the more chronic effects of cathepsin G to activate caspase-3 and cleave AKT; FAK cleavage is delayed and attenuated (but not completely blocked) by PP1. The effect of PP1 to inhibit the activation of caspase-3 and cleavage of AKT (and to a lesser extent FAK) suggests that these events are related. Consistent with this formulation, levels of AKT and FAK remain constant in cardiomyocytes exposed to cathepsin G in the presence of Z-DEVD-FMK (a cell-permeable general caspase inhibitor). These results implicate Src kinases and caspase-3 in the pathway for AKT and FAK cleavage during cathepsin G treatment. Significantly, Fig. 7B shows that the effects of PP1 and Z-VAD-FMK to inhibit the proteolysis of signaling proteins delays the kinetics of cell detachment, but neither is sufficient to completely abrogate the morphologic changes induced by cathepsin G.

**DISCUSSION**

This study provides the first evidence that neutrophil cathepsin G acts as a potent agonist for cardiomyocytes. Cathepsin G elicits a spectrum of acute signaling responses that in part mimic cardiomyocyte activation by thrombin; both cathepsin G and thrombin promote phosphoinositide hydrolysis, activate ERK and p38 MAPK cascades, stimulate AKT, and inhibit contractile function. The acute stimulatory effects of thrombin have been attributed to proteolytic activation of PAR-1; recent studies (19) identify PAR-4 as an additional molecular target for the action of thrombin in cardiomyocytes. However, there are striking differences between the acute signaling responses induced by maximally stimulatory concentrations of conventional PAR agonists (thrombin, SFLLRN, or AYPGKF) versus cathepsin G that raise serious doubts that a known PAR mediates the action of cathepsin G in cardiomyocytes. First, thrombin and the PAR-1 agonist peptide promote brisk/robust increases in IP3 and IP2, which are followed by a more sustained increase in the level of IP1; this response is characteristic of the conventional G protein-coupled receptor-dependent signaling pathway leading to the activation of phospholipase C.
In contrast, cathepsin G promotes the preferential accumulation of IP$_3$ and IP$_2$, with atypically slow kinetics and little associated accumulation of IP$_1$. To the best of our knowledge, this pattern of phosphoinositide hydrolysis does not correspond to any conventional G protein-coupled receptor signaling response reported previously. The effects of PAR agonists and cathepsin G on intracellular calcium also differ. PAR-1 activation with thrombin or SFLLRN results in a brisk rise in intracellular calcium. In contrast, intracellular calcium does not change during acute incubations with cathepsin G (although an effect of prolonged cathepsin G stimulation to alter intracellular calcium secondary to cathepsin G-induced changes in cell morphology are possible and would not be excluded by these experiments). These differences in PAR-1 agonist versus cathepsin G actions provided the first hints that the cardiac actions of cathepsin G might not simply be attributable to proteolytic cleavage of a PAR. The distinct morphology of cardiomyocytes exposed to conventional PAR agonists (where there is enhanced sarcomeric organization and hypertrophy) versus cathepsin G (where activation of executioner caspases leads to cleavage of structural and signaling proteins, myofibrillar disarray, and cardiomyocyte detachment from underlying substrate) provided further evidence that thrombin and cathepsin G act via distinct molecular targets.

Several complementary strategies were used to explore further a potential role for PARs in the actions of cathepsin G. In agreement with previous studies suggesting that PAR-1 can function as a substrate for cathepsin G cleavage under certain circumstances in heterologous expression systems, but that PAR-1 does not likely mediate cathepsin G responses in the physiologic context, studies reported herein demonstrate that PAR-1 expression is not required for cathepsin G signaling responses in cardiomyocytes. Phospholipase C and p38 MAPK activation by cathepsin G is equivalent in wild-type and PAR-1/H11002/H11002 cardiomyocytes. A previous study implicated PAR-4 in the cellular actions of cathepsin G in human platelets (and inferred that PAR-4 acts as a cathepsin G receptor in human PAR-4-expressing Xenopus oocytes and lung fibroblasts derived from PAR-1/H11002 mice) (10). The experimental evidence to support the conclusion that human PAR-4 supports oocyte activation by cathepsin G is compelling; cathepsin G triggers calcium release from oocytes that express human PAR-4 but not from oocytes that express a PAR-4 mutant (glycine to proline substitution at position 1 of the agonist peptide) that is rendered resistant to thrombin cleavage but remains responsive to AYPGKF. How-

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**Fig. 9.** Cathepsin G activates caspase-3 and promotes FAK and AKT cleavage; role of Src kinases. Neonatal rat cardiomyocyte cultures were pretreated for 30 min with vehicle, PP1 (10 μM), or Z-VAD-fmk (100 μM) followed by incubation in the absence or presence of 400 nM cathepsin G for the indicated intervals. Immunoblot analysis was with antibodies that recognize the caspase-3 cleavage product (a polyclonal antibody to N-terminal residues adjacent to Asp$^{175}$), AKT, FAK (a monoclonal antibody to residues 355–533, which includes part of the kinase domain), and ERK. A longer exposure is provided for optimal visualization of the FAK cleavage product. Illustrated immunoblots are representative of results obtained in two to three separate cultures.

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**Fig. 10.** Cathepsin G-dependent activation of AKT and stimulation of phosphoinositide hydrolysis; role of Src kinases. Neonatal rat cardiomyocyte cultures were pretreated with vehicle or PP1 (10 μM) and then incubated in the absence or presence of cathepsin G (for the indicated intervals in A or for 30 min in B). A, measurements of AKT, p38 MAPK, and ERK activation/phosphorylation were performed by Western blot analysis (as described in Fig. 2; total AKT, p38 MAPK, and ERK protein levels do not change under these conditions, data not shown). B, IP$_2$ accumulation (expressed as counts/min) was measured as described in Fig. 1; results are mean ± S.E. of triplicate determinations from 3 separate experiments.
ever, the experimental evidence implicating PAR-4 cleavage as the mechanism mediating cathepsin G actions in fibroblasts is more tenuous. The experimental findings are limited to the observation that cathepsin G mobilizes intracellular calcium in PAR-4-transfected, but not untransfected, lung fibroblasts (10). Direct evidence that cathepsin G cleaves PAR-4 has never been presented. This is pertinent, as a working model that attributes the actions of cathepsin G to an activating cleavage of endogenous PAR-4 does not accommodate recent results (13) obtained in murine platelets, where cathepsin G does not trigger aggregation (suggesting that cathepsin G does not proteolytically activate murine PAR-4). Experimental data reported herein also are more consistent with a PAR-4-independent mechanism for cathepsin G action. Specifically, our studies demonstrate that expression of human PAR-4 confers responsiveness to thrombin and AYPGKF but not to cathepsin G; cathepsin G does not mimic the actions of thrombin to activate proximal signals (IP accumulation, ERK, p38 MAPK, and AKT) in PAR-1−/− fibroblasts that express PAR-4. The further observation that cathepsin G-treated cells remain fully responsive to the stimulatory actions of thrombin argues that cathepsin G also does not cleave PAR-4 at a site that amputates the tethered ligand domain. These results raise serious doubts that PAR-4 cleavage significantly contributes to the actions of cathepsin G. The differences in cathepsin G responsiveness between this and the previous study are not likely to be attributable to cell-specific differences in post-translational processing of PAR-4, as both studies were performed on clonal derivatives of PAR-1−/− lung fibroblasts that stably express human PAR-4. Rather, it is reasonable to speculate that this study (which measures phospholipase C activation, a proximal element in the signaling pathway) provides a more sensitive measure of PAR-4 activation. Hence, the cathepsin G-dependent rise in intracellular calcium identified in the previous study using PAR-4 expressing PAR-1−/− lung fibroblasts could reflect the secondary consequence of cathepsin G-induced changes in cell morphology and/or adherence to substrate; such a calcium response would preferentially be manifest in cells that overexpress PAR-4, which display the most conspicuous morphologic changes during incubation with cathepsin G. In this context, the observation that cathepsin G induces pronounced morphologic changes in cells that express PAR-4, but exerts little effect in cells that express PAR-1, suggests that PAR-4 activation leads to changes in cell surface receptor and/or adhesion molecule expression that render cells particularly susceptible to the actions of cathepsin G (and that these cell types constitute a particularly useful model to explore the molecular basis of the actions of cathepsin G).

The cathepsin G-induced changes in cardiomyocyte morphology described in this study are very reminiscent of the changes described previously in endothelial cell monolayers, where an initial effect of cathepsin G to disrupt intercellular contacts and expose the underlying extracellular matrix (impairing the barrier properties of the monolayer and exposing a potentially thrombogenic surface) is followed by cleavage of extracellular matrix components and de-endothelialization (25). The mechanisms underlying the cellular actions of cathepsin G are still incompletely understood. There is some evidence that cathepsin G promotes tissue remodeling by directly degrading components of the matrix. However, cathepsin G also can act indirectly by cleaving and activating matrix-degrading metalloproteinases (MMPs, which are stored in the extracellular space as latent proenzymes). Cathepsin G-induced cleavage of MMP2 has been implicated as a mechanism for neutrophil-mediated tissue remodeling and injury in the microvasculature (32). Studies in progress identify a similar effect of cathepsin G to release cleaved/activated MMP-2 from cardiomyocyte cultures; studies to define the precise mechanism(s) for MMP activation and the role of MMPs in the cardiac actions of cathepsin G are ongoing.2

This study provides novel evidence that neutrophil-derived cathepsin G can induce cardiac injury by triggering detachment-induced apoptosis (anokisia) and the activation of executioner caspases. The targets for caspase-dependent cleavage in cardiomyocytes exposed to cathepsin G include critical components of the adhesion complex that supports normal cell-matrix interactions as well as signaling proteins necessary for cell survival, suggesting a mechanism that contributes to cathepsin G-induced injury. Caspase-mediated cleavage of FAK contributes to the execution of the apoptosis program both by interrupting survival signals from the extracellular matrix and by promoting disassembly of the focal adhesion complex (27). The ordered cleavage of FAK into discrete polypeptide fragments results in a decline in overall levels of full-length FAK and the accumulation of C-terminal FAK fragments that structurally resemble the FAK-related non-kinase FRNK (a naturally occurring FAK inhibitor that prevents FAK localization to site of integrin engagement, decreases FAK phosphorylation, and inhibits FAK-mediated responses); both events would block the formation of focal adhesions and liberate cells from the anti-apoptotic signals generated by extracellular matrix attachment. Similarly, caspase-mediated cleavage of AKT is presumed to contribute to apoptosis by reducing levels of AKT below a critical threshold, such that key survival pathways are disabled (29, 33). Caspase-mediated cleavage of the Src family kinase Fyn has been identified as a mechanism to increase its activity and also might be predicted to mediate the cardiac actions of cathepsin G (30). Finally, a recent study (34) identifies caspase-3-dependent cleavage of certain myofibrillar proteins (including α-actin, α-actinin, and troponin T) as a mechanism that impairs contractility and limits the functional reserve of pre-morbid cardiomyocytes. Myofibrillar disarray and troponin T cleavage is detected in cathepsin G-treated cardiomyocytes (Fig. 7 and data not shown), suggesting that this also may contribute to the pathogenesis of cathepsin G-dependent actions in the heart.

The designation of cardiomyocytes as a cathepsin G-responsive cell type indicates that it is a protease other than thrombin and trypsin can contribute to changes in cardiomyocyte structure and function in a potentially clinically relevant manner. Although most protease actions have been ascribed to cleavage of PARs, these studies suggest a novel mechanism for entree into pathways that modulate cardiomyocyte function. The effects of cathepsin G on adhesion-dependent signaling would be particularly important in areas of interstitial inflammation, such as the border zone adjacent to a myocardial infarction, where neutrophil infiltration leads to important local changes in gene expression, cardiomyocyte hypertrophy, contractile dysfunction, and apoptosis. Activated neutrophils are believed to contribute to tissue remodeling (and influence reperfusion injury in neighboring cardiomyocytes) via the release of oxygen-derived free radicals, eicosanoids, cytokines, and proteolytic enzymes. However, the relative importance of individual cytotoxic mediators has been difficult to resolve. These studies, in the context of recent evidence that LEX032 (a recombinant serine protease inhibitor of neutrophil cathepsin G and elastase) reduces myocardial necrosis and affords cardioprotection during in vivo ischemia/reperfusion in the rat (35), suggest that neutrophil-derived proteases such as cathepsin G may be a particularly important mediator of cardiac injury.

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