Proteinase-activated Receptors, Targets for Kallikrein Signaling

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Serine proteinases like thrombin can signal to cells by the cleavage/activation of proteinase-activated receptors (PARs). Although thrombin is a recognized physiological activator of PAR1 and PAR3, the endogenous enzymes responsible for activating PAR2 in settings other than the gastrointestinal system, where trypsin can activate PAR2, are unknown. We tested the hypothesis that the human tissue kallikrein (hK) family of proteinases regulates PAR signaling by using the following: 1) a high pressure liquid chromatography (HPLC)-mass spectral analysis of the cleavage products yielded upon incubation of hK5, -6, and -14 with synthetic PAR N-terminal peptide sequences representing the cleavage/activation motifs of PAR1, PAR2, and PAR3; 2) PAR-dependent calcium signaling responses in cells expressing PAR2, PAR3, and PAR1 and in human platelets; 3) a vascular ring vasorelaxation assay; and 4) a PAR4-dependent rat and human platelet aggregation assay. We found that hK5, -6, and -14 all yielded PAR peptide cleavage sequences consistent with either receptor activation or inactivation/disarming. Furthermore, hK14 was able to activate PAR1, PAR2, and PAR3 and to disarm/inhibit PAR4. Although hK5 and -6 were also able to activate PAR2, they failed to cause PAR4-dependent aggregation of rat and human platelets, although hK14 did. Furthermore, the relative potencies and maximum effects of hK14 and -6 to activate PAR2-mediated calcium signaling differed. Our data indicate that in physiological settings, hKs may represent important endogenous regulators of the PARs and that different hKs can have differential actions on PAR1, PAR2, and PAR4.

Proteinase-activated receptors (PAR1−4)3 compose a unique family of four G-protein-coupled cell surface receptors for certain proteinases (1–9). Proteolytic cleavage within the extracellular N terminus reveals a tethered ligand that binds to the extracellular receptor domains to initiate cell signaling (5, 6, 9, 10). Proteinases that activate PARs include coagulation factors, enzymes from inflammatory cells, and proteinases from epithelial cells and neurons. These enzymes, generated and released during injury and inflammation, can cleave and activate PARs on many cell types from a variety of species (humans, rats, and mice) to regulate the critically important processes of hemostasis, inflammation, pain, and tissue repair. Other proteinases that cleave PARs downstream of the N-terminal tethered ligand sequence disable the receptors for further proteolytic activation, thus abrogating PAR signaling. It is of considerable interest to identify the proteinases that activate and/or disable PARs, in view of the emerging role that these receptors can play in diseases such as asthma, arthritis, inflammatory bowel disease, and cancer (5–7).

In some systems, the proteinases that activate PARs have been established. For example, in the circulatory system, PAR1, PAR3, and PAR4 are recognized physiological targets for thrombin (4), which does not efficiently activate PAR2. How-

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3 The abbreviations used are: PAR, proteinase-activated receptor; r, rat; h, human; hK, human kallikrein protein; KNRRK, Kirsten virus-transformed normal rat kidney cells; KNRRK PAR2, rat PAR2-expressing KNRRK cells; KNRRK PAR3 (R36A), KNRRK cells expressing a tryptysin-resistant rat PAR3 mutant; L-NAME, Nω-nitro-L-arginine methyl ester; Trp, tryptysin; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; AMB, amino-4-methylcoumarin; MALDI, matrix-assisted laser desorption ionization; LC-MS, liquid chromatography/mass spectrometry; PBS, phosphate-buffered saline.
ever, PAR2 can be activated by the complex of tissue factors VIIa and Xa (11). In the gastrointestinal tract, trypsin (presumably, trypsin-1 or -2 in humans) can activate PAR2 on enterocytes (12). Mast cell tryptase, which in humans can be released in the vicinity of sensory nerves, is another candidate enzyme that may regulate PAR2, in vivo (13–15). Although a number of other serine proteinases are known to trigger PAR2 signaling (7), the endogenous enzymes responsible for activating PAR2 in settings other than the gastrointestinal tract where trypsin can activate PAR2 are unknown. Moreover, apart from the observations that elastase and chymase can disable PAR2 (16, 17), little is known about the proteinases that can inactivate either this receptor or other members of the PAR family. As outlined above, PAR signaling has been implicated in a variety of physiological processes, including the regulation of vascular and gastric smooth muscle contractility, inflammation, nociception, cell adhesion, metastasis, proliferation, and apoptosis, as well as in the delayed immune response (7–9, 18, 19).

Tissue kallikreins compose a large family of secreted serine proteinases with trypsin or chymotryptic activity (20, 21). These enzymes, which share a high degree of genomic and protein sequence homology, are present in many mammalian species. In humans, their genes are tandemly localized on chromosome 19q13.4. The human kallikreins are abundantly expressed in groups in many tissues, often in a sex-steroid hormone-dependent manner, and they are up-regulated in disease states. For example, hK5, -6, and -14 are found at increased levels in the serum and/or ascites fluid of ovarian cancer patients (22–26) as well as in the serum of breast cancer patients (23, 26). Kallikreins are also highly expressed at sites of inflammation (27, 28). However, despite their widespread expression in diseased tissues, the mechanisms whereby this enzyme family regulates cellular function are not clear. Proteins of the extracellular matrix, pro-urokinase-plasminogen activator, kininogens, growth factor precursors (and binding proteins), and other kallikreins are potential targets of kallikrein proteolysis during cancer progression (20, 21, 29–31). Such targets may well explain some but by no means all of the physiological actions of kallikreins, particularly in the setting of cancer. PARs are also up-regulated in cancer and inflammation, and PARs have been proposed as substrates for kallikreins (20). However, the ability of kallikreins to cleave and activate or disable PARs has not as yet been examined.

We hypothesized that the human kallikrein (hK) family of serine proteinases regulates signal transduction by cleaving the PARs. We tested this hypothesis by using the following: 1) an HPLC-mass spectral analysis of the cleavage products yielded upon incubation of hK5, -6, and -14 with peptides corresponding to the N-terminal tethered ligand domains of PAR1, PAR2, and PAR4; 2) PAR-dependent calcium signaling responses in cell lines expressing PAR1, PAR2, and PAR4 and in isolated human platelets; 3) a vascular ring vasorelaxation assay using tissues from rats and both wild-type and PAR2 null mice; and 4) a PAR4-dependent rat and human platelet aggregation assay. hK14 was selected as a “prototype” trypsin-like kallikrein, because of its particularly wide tissue distribution. We focused on the ability of hK14 to regulate PAR2 in particular, because of the well recognized susceptibility of this PAR family member to trypsin-like serine proteinase activation. We also evaluated hK14 for its ability to cleave/activate PAR1 and PAR4, and for comparison, we determined whether hK5 and -6 might also regulate PAR1, PAR2, and PAR4. To determine whether PARs from species other than humans might also be subject to kallikrein regulation, we used cell and tissue preparations from rats and mice in addition to evaluating hK action on human PAR1, PAR2, and PAR4.

**MATERIALS AND METHODS**

**Peptides and Other Reagents**

All peptides were synthesized by standard solid phase methods by the Peptide Synthesis Facility at the Faculty of Medicine, University of Calgary. Peptide composition and purity were ascertained by HPLC analysis, amino acid analysis, and mass spectrometry. Stock solutions (about 1 mM) were prepared in 25 mM HEPES buffer, pH 7.4, and peptide concentrations were verified by quantitative amino acid analysis. Porcine trypsin (catalog number T-7418; 14,900 units/mg), phenylephrine, acetylcholine, L-NAME (the nitric-oxide synthase inhibitor, \( N^\omega \)-nitro-\( L \)-arginine methyl ester), and the calcium ionophore A23187 were from Sigma. High activity human thrombin (catalog number 605195; lot B37722; 3,186 units/mg) was from Calbiochem. The calcium indicator Fluo-3 acetoxymethyl ester was from Molecular Probes (Eugene OR), and the PAR1 antagonist SCH 79797 was from Tocris Bioscience (Ellisville, MO).
The agonists utilized in the following experiments and their symbols used in the figures are summarized in Table 1.

**Production, Purification, and Activation of Recombinant hK5, -6, and -14**

Recombinant hK6 containing the enterokinase-specific recognition signal (Asp)₄Lys, in order to be sensitive to full activation by enterokinase, was produced in a baculovirus system, as described previously (32). Protein was stored in 10 mM sodium acetate, 0.01% Tween 20, pH 5.2, to prevent protein degradation. hK5, expressed in *Pichia pastoris*, was purified and activated, as described (33). hK14, expressed in yeast as the active form of the enzyme, was purified to yield catalytically active enzyme (>80% active) as described previously (26), with some modifications. In brief, concentrated yeast supernatant, diluted 1:2 with running buffer (10 mM MES, pH 5.3), was loaded onto a 5-ml HiTrap SP HP-Sepharose cation exchange column (Amersham Biosciences). The column was eluted with 10 mM MES buffer, pH 5.3, with a linear gradient of 0–1 M KCl. Fractions were analyzed by hK14-specific enzyme-linked immunosorbent assay, pooled, and concentrated 10 times in 10 mM MES, pH 5.3. The concentration of the purified catalytically active recombinant protein was determined by enzyme-linked immunosorbent assay and total protein assay. The purity of the protein (>90%) was verified by SDS-PAGE.

**Standardizing Kallikrein Enzyme Activity**

To facilitate direct comparisons between the different kallikrein preparations for their ability to activate or disarm the PARs, the concentration of each hK preparation (hK5, -6, and -14) was standardized for its enzymatic activity in terms of its “trypsin-like equivalents,” as follows. The enzymatic activity of kallikreins 5 and 6 was estimated essentially as described previously (33, 34), using the fluorescent synthetic substrate t-butoxycarbonyl-Val-Pro-Arg-7-amino-4-methylcoumarin (AMC) (Bachem, King of Prussia, PA). In brief, each kallikrein was incubated at 37 °C, in a microtiter plate, with the optimal activity assay buffer and varying concentrations (within the linear range) of fluorescent substrates in a final volume of 100 μl. The initial rate of AMC release was measured on a Wallac Victor fluorometer (PerkinElmer Life Sciences) set at 355 nm for excitation and 460 nm for emission. The fluorescence values of enzyme-free reactions were used as the negative controls, and background fluorescence was subtracted from each value. All experiments were performed in triplicate. A standard curve with known concentrations of AMC was used to calculate the rate of product formation. Kinetic analysis was done by nonlinear regression analysis using the Enzyme Kinetics Module 1.1 (Sigma Plot, SPS, Chicago, IL). For hK6, the assay was the same as for hK5, but the buffer was changed to 50 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, 0.01% Tween 20, pH 7.3. The conditions for estimating the enzymatic activity of hK14 were identical to those described for hK5, except that t-butoxycarbonyl-Val-Ala-Arg-7-Amino-4-methylcoumarin was used as substrate. To standardize the activities of different kallikrein preparations, the activity of each hK enzyme batch was expressed relative to the activity of a standard concentration of pure trypsin (5 nM or 2.5 units/ml), measured using the same AMC substrate employed for assessing the kallikrein activity. For molar calculations, 1 unit/ml trypsin enzyme activity was taken as 2 nM. Thus, the kallikrein concentrations shown as absissa values in the figures are shown as units of “trypsin-like equivalents of activity,” based on the above assays. This approach was used for a direct comparison between the proteinases, since each kallikrein preparation contained active enzyme along with a small (<10%) but variable proportion of inactive enzyme. Thus, any estimate of the enzyme EC₅₀ based on protein content alone would have been misleading. Therefore, the data shown in the figures reflect information specifically related to the active enzyme in each hK preparation.

**Mass Spectral Analysis of Peptide Proteolysis Products**

The following synthetic sequences derived from human and rat PAR₁, PAR₂, and PAR₄ were selected for the proteolysis studies (critical tethered ligand-activating sequences are underlined) (Table 2): (a) hPAR₁, NATLDPRSFLLRNPNDKYE; (b) hPAR₂, N-acetyl-GTNRSKGRSLIKGVDTGHTVGKTGVTGVT-amide; (c) hPAR₄, GDDTSPSLAPRPGYPGVQ; (d) rPAR₁, GPNSKGRSLI-GRLDTTPYGGC (non-PAR residues, YGGC, added for affinity-column coupling and ¹²⁵I labeling); and (e) rPAR₄, LNESKSDPKP-NPRGFPKGP-amide.

These peptides at a concentration of 100 μM were incubated with kallikreins 5 (3.1 units/ml), 6 (6.08 units/ml), 14 (4.3 units/ml), or trypsin (3.5 units/ml) for varying times up to 30 min at 37 °C. The incubation buffers were identical to the buffers used for the measurements of enzymatic activities. Reactions were terminated either by rapid freezing in liquid nitrogen or by the addition to the proteolysis sample of 2 volumes of a “stop solution” comprising 50% acetonitrile and 0.1% trifluoroacetic acid in water. Samples were either subjected immediately to HPLC analysis, with collection of the E₂₁⁴ peak fractions, or were stored at −80 °C for further processing. The cleavage products were identified by three approaches as follows. 1) HPLC separation and isolation of the proteolysis products was followed by mass spectral MALDI identification of the peptide fragments in the quantified HPLC peaks. The HPLC analysis did not provide data for proteolysis products present in low abundance, so as not to be detected by the elution profile monitored by absorbance at 214 nm. 2) Combined liquid chromatography-mass spectral (LC-MS) analysis (Agilent HP 1100 Nanoflow System) was followed by linear ion trap quadrupole analysis (Thermo Electron Corp.) of the entire proteolysis mixture without prior HPLC separation. For this second approach, the relative abundance of the LC-separated constituents was not determined. 3) Matrix-assisted laser desorption ionization (MALDI) mass spectrometry of the complex hydrolysis reaction mixture was performed with computer-assisted deconvolution of the mass spectra. Like the LC-MS approach, this method did not provide information about the relative abundance of the peptides detected. Analyses were done either at the proteomic facilities of the Samuel Lunenfeld Research Institute (Mount Sinai Hospital, Toronto, Ontario, Canada) or in the Southern Alberta Mass Spectrometry Facility at the University of Calgary, Faculty of Medicine (Calgary, Alberta, Canada).
Kallikrein Signaling via Proteinase-activated Receptors

Cell Culture and PAR-expressing Cell Lines

Kirsten virus-transformed normal rat kidney (KNRK) epithelial cell lines expressing either wild-type rat PAR2 (KNRK-PAR2) or the trypsin-resistant rat PAR2(R36A) variant were validated previously for studies of receptor activation (35) and were therefore used to evaluate the PAR2-activating properties of the kallikreins by the methods described previously (35, 36). Human embryonic kidney cells (HEK293) that express the SV40 T-antigen were kindly provided by Dr. Jonathan Lytton, University of Calgary, Faculty of Medicine, Calgary, Alberta, Canada. The human HEK293 cells that constitutively express both PAR1 and PAR2 were grown as for the KNRK cells, but in the absence of genetin, as outlined for the PAR-mediated calcium signaling procedure described previously (36). A rat PAR4-expressing HEK cell line HEKrPAR4 was generated from a receptor clone kindly provided by Dr. W. A. Hoogerwerf, Galveston, TX (37). In brief, using the rat PAR4 clone, an N-terminal Myc and C-terminal herpes simplex virus tag were added by PCR employing the primers: forward, 5′-GAGCCGAAGGCTGATCGCAAGAGAGGCTGCGGCGGGAATGCGGAGCAGCAGCC-3′, and reverse, 5′-ATCTCGAGTCATCAGCAGTCCCTGGGGTGTCCTGCGGGGCGTCGAGAAGGTGAGAGGACAAATG-3′, using standard techniques. The modified cDNA was then subcloned into the vector pcDNA3.1-Igκ neo (38) to yield the expression vector pcDNA3.1-Igκ myc-rPAR4HSV. This vector was used to transfect the HEKFLP cell line (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s guidelines. After 2 days, the cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 200 μg/ml G418 sulfate, and 100 μg/ml Zeocin for 3 weeks. Individual foci were expanded and assayed for expression by immunofluorescence. After selection, the rat PAR4-expressing HEK cells (HEKrPAR4) were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 100 μg/ml G418 sulfate, 100 μg/ml Zeocin. A vector control cell line was similarly engineered and propagated. All cells were subcultured by dissociation in an isotonic EDTA/saline solution, pH 7.4, without the use of trypsin, to avoid cleavage of PARs.

Site-targeted Receptor Antibodies and Immunohistochemical Procedures for Monitoring PAR2 Cleavage/Activation

To assess morphologically the ability of the hKs to cause the activation or “disarming” of PAR2, we made use of a rat PAR2 expression system (receptor expressed in the KNRK cells) along with two rat PAR2-targeted antisera that recognize either the entire cleavage-activation sequence (B5) (35) or only the pre-cleavage sequence (SLAW-A) (35, 39, 40). The use of these two antisera to document receptor activation or disarming has been validated for rat PAR2 both with cells grown in vitro and in tissues monitored in vivo (35, 39–41). Unfortunately a comparable validation of similar antisera to monitor the activation/disarming of human PAR2 has not yet succeeded. With this approach, activation of the receptor without a downstream cleavage that would remove the tethered ligand entirely results in a disappearance of reactivity with the SLAW-A antibody but retention of reactivity with B5. Alternatively, cleavage of the receptor downstream of the tethered ligand sequence abolishes the reactivity of the cells with both the B5 and SLAW-A antisera. Polyclonal rabbit anti-PAR2, antisera (B5 and SLAW-A) were raised in rabbits and used as described elsewhere (12, 35, 39). The antibodies were generated against peptides that correspond to sequences representing the potentially antigenic epitopes either in the pre-cleavage domain of wild-type rat PAR2 (for SLAW-A, SLAWLGGPPNSKGR) or in the tethered ligand cleavage/activation site (denoted by “/”) of rat PAR2 (for B5, GPNSKGR/SLIGRLDTTP). As mentioned, one or both of these epitopes can potentially be lost from the cell surface upon cleavage/activation of the receptor by proteinases. The removal of the N-terminal antigenic determinants by kallikreins or by trypsin was assessed by monitoring the decrease of cell surface reactivity with the SLAW-A and B5 antibodies (39, 40, 41). Neither the B5 nor the SLAW-A antibodies react with KNRK cells transfected with vector alone. Furthermore, the reactivity of both antibodies with PAR2-expressing KNRK cells is abolished by pre-absorption with the immunizing peptide (39, 41). To monitor the ability of hK5, -6, and -14 to cleave PAR2 at or beyond the tethered ligand activation site, KNRK-rPAR2 cell suspensions were treated or not for 5 min at room temperature with enzyme concentrations that had been documented to activate rat PAR2 in the calcium signaling assay. The effects of the kallikreins were compared with the actions of trypsin (20 units/ml; about 40 nM). For immunocytochemical morphometric analysis, enzyme-treated cells were spun onto a glass microscope slide, using a Shandon cytospin apparatus (Shandon Scientific, Cheshire, UK), washed with PBS (5 s), fixed in 5% formaldehyde, and 0.05% Tween 20 in PBS (5 s), air-dried, and mounted onto slides with 90% glycerol, 10% Tris (1M, pH 8). The fluorescence was visualized using a Leica microscope (Cambridge, UK), and images were analyzed using the software Image J. For experiments done on three separate occasions with independently cultured cell samples, images representative of five or more different representative fields of view were recorded. The fluorescence intensity was then measured at five different points along the cell surface in each of the five or more images, and averages were taken. The ratio of the fluorescence values resulting after proteolytic cleavage by an enzyme with respect to the fluorescence of nontreated cells was used to determine the percentage of receptors not cleaved. The percent cleavage was determined by subtracting the above number from 100. The numbers reported in this study corre-
spond to the means and S.E. of measurements obtained from the three different experiments. A control cell population of KNRK cells expressing wtPAR2 regularly scored >80–90% positive; vector-transfected cells or stained preparations in which the antisera had been pre-adsorbed with the immunizing peptides were routinely negative, as described previously (35, 39, 41). In summary, disappearance of SLAW-A reactivity, with retention of B5 reactivity, reflected cell activation by cleavage at the R/S tethered ligand cleavage-activation site of PAR2. Alternatively, disappearance of cellular reactivity toward the B5 antiserum (the SLAW-A reactivity would also be absent) indicated disarming of the receptor by cleavage downstream from the tethered ligand sequence as observed previously for neutrophil elastase (16). The quantitative morphometric analysis used to quantify receptor activation-disarming has been validated for both cultured cells and intact tissues (16, 35, 39, 40).

**Measurements of PAR-regulated Calcium Signaling, Receptor Desensitization Protocol, and Evaluation of Receptor Disarming/Inhibition**

**Calcium Signaling**—Cell lines (grown to about 85% confluency and disaggregated with calcium-free isotonic phosphate-buffered saline containing 0.2 mM EDTA) and rat and human platelets (harvested from platelet-rich anticoagulated plasma) were incubated for 25 min at room temperature with Fluo-3 acetoxymethyl ester (final concentration, 22 μM) along with 0.4 mM sulfinpyrazone. Cells were washed twice by centrifugation (using the following buffer without calcium) and resuspended in the calcium signaling buffer: 150 mM NaCl, 3 mM KCl, 1.5 mM CaCl2, 20 mM HEPES, 10 mM dextrose, and 0.25 mM sulfinpyrazone, pH 7.4. Fluorescence was measured at 24 °C with an excitation wavelength of 480 nm and an emission recorded at 530 nm using an Aminco Bowman Series 2 Luminescence spectrometer (ThermoSpectronic Model FA354, Spectronic Unicam, Rochester, NY). The fluorescence signals caused by the addition of test agonists (trypsin, kallikreins, or PAR-activating peptide, added to 2 ml of a cell suspension of about 3 × 106 cells/ml) were compared with the fluorescence peak height yielded by replicate cell suspensions treated with 2 μM ionophore A23187. This concentration of A23187 was at the plateau of its concentration-response curve for a fluorescence response in Fluo-3-loaded cultured cells. Under these conditions, the calculated values for intracellular calcium in the HEK and KNRK cells were ~30 nm under basal conditions and about 340 nm upon exposure to A23187 (42, 43). Previous work (36, 44) has shown that the fluorescence response of a cell preparation, as a percentage relative to the signal generated by 2 μM A23187, is a valid reference standard for the comparative determination of calcium signals for all PAR agonists. The cellular calcium response was therefore recorded as a percentage of the response generated by 2 μM ionophore A23187 (% A23187) (36). Concentration-effect curves for hK14 and hK6 were obtained by measuring the calcium signals generated by increasing enzyme concentrations in replicate cell suspensions (2 ml). Values at each enzyme concentration represent the averages (± S.E.: error bars above average values) of measurements done with at least three cell replicates derived from three or more independently grown cultures of PAR-expressing cells.

**Selective Receptor Desensitization Protocols**—To monitor the cellular response to an individual PAR in the HEK cells that express multiple PARs (PAR, and PAR2 in background HEK cells; PAR1, PAR2, and PAR3 in HEK PAR2 cells), we made use of a selective receptor desensitization protocol (36). Cells were exposed to a PAR agonist at a supramaximal concentration (to desensitize a selected PAR fully) or vehicle (control) and were then challenged with a second agonist after a 5–10 min interval, sufficient for the re-filling of the intracellular calcium stores (e.g., see Ref. 36). To assess the ability of hK5, -6, and -14 to activate one or both of PAR1 and PAR2, the HEK cells were first desensitized by exposure to either the PAR1AP, TFLLR-NH2, or the PAR2AP, SLIGRL-NH2 (or both), followed by the addition of hK5, -6, or -14. The persistence of a calcium signal in response to the hKs after desensitization of PAR1 by TFLLR-NH2 demonstrated the activation of PAR1 by the proteinase, and the persistence of a calcium signal after desensitization caused by the PAR2AP, SLIGRL-NH2, indicated an activation of PAR2. Furthermore, the complete disappearance of an hK-induced calcium signal after pre-desensitizing the HEK cells with TFLLR-NH2 and SLIGRL-NH2 in combination (100 μM each) or with 200 μM of the PAR nonselective agonist SFLLR-NH2 would serve to indicate that the calcium signal caused by the proteinase did not result from the activation of a non-PAR mechanism.

**Evaluation of Receptor Disarming/Inhibition Using the Calcium Signaling Assay**—Proteolytic removal of the tethered ligand by cleavage downstream from the activation domain can disarm a PAR, so as to inhibit its proteolytic activation but still leave the cell sensitive to activation by a PAR-activating peptide (16, 17, 36). In this process the proteinase amputates the receptor-activating sequence but may possibly also bind tightly in a noncatalytic mode to the receptor cleavage site, thereby inhibiting activation by other proteinases. To determine whether hK14 disarms/inhibits PAR1 for activation by thrombin, we exposed wild-type HEK cells for 10 min to increasing concentrations of hK14, starting with hK14 concentrations determined to result in a minimal activation of PAR1. In the continued presence of hK14, the cells were then challenged either with a PAR1-activating concentration of thrombin (0.5 units/ml; 5 nM) or with a PAR1-activating concentration of TFLLR-NH2 (5 μM). The thrombin-induced and peptide-induced calcium signals measured after hK14 exposure were compared (% control) with the “control” calcium signal caused by these agonists in the absence of hK exposure in the same cell preparation. The diminished “residual” PAR1 response to thrombin represents both disarming and desensitization. As a variation on this protocol, cells first pretreated or not with hK14 for 10 min were washed free of enzyme and resuspended in calcium assay buffer for the measurement of thrombin-activated and peptide-activated calcium signaling. This protocol ensured that the residual hK14 in the incubation medium did not block or otherwise inhibit thrombin action. Again a reduced thrombin response in the washed, hK14-treated cells, with retention of the signal caused by TFLLR-NH2, reflected a disarming of PAR1. Alternatively, the diminished response to the peptide after hK14 treat-
ment represents only receptor desensitization and not disarm-
ing (36). In the same cell preparation, the PAR1 calcium signal
trigged by hK14 itself (monitored after desensitization of
PAR2 by prior treatment with SLIGRL-NH2) was also meas-
ured. Because of the complexity of this procedure (which
requires the use of nondenaturing concentrations of hK14 to
observe receptor disarming), it was not possible to do compa-
parable experiments to measure with confidence the disarming by
hK14 of either PAR2 or PAR4 for activation by either trypsin
(PAR2) or thrombin (PAR4).

**Bioassay Procedures**

*Platelet Isolation and Aggregation Assay—* Venous anticoag-
ulated blood (0.5 ml of 3.4% w/v trisodium citrate per 10 ml of
blood) was collected from human male volunteers and from
male Sprague-Dawley rats (200–300 g, inferior vena cava punc-
ture) by institutionally approved protocols. A platelet-rich
plasma suspension was obtained by centrifugation (900
rpm; 150 × g_{max}) at room temperature for 15 min (r_{max} = 170
mm). The platelet-rich plasma supernatant was withdrawn for
the preparation of washed platelets. To prepare washed plate-
lets, the platelet suspension was supplemented with 0.8 μM
prostaglandin I2 (300 ng/ml), harvested by centrifugation
(1,800 rpm; 620 × g_{max} for 10 min at room temperature), and
resuspended in prostaglandin I2 (0.8 μM)-supplemented calci-
um-free Tyrode’s buffer, pH 7.4, of the following composition:
NaCl (136 mM), KCl (3 mM), NaHCO3 (12 mM), NaH2PO4 (0.4
mM), MgCl2 (1 mM), and glucose (6 mM). Platelets were again
collected by centrifugation (1400 rpm; 370 × g_{max} for 10 min at
room temperature) and resuspended in calcium-free Tyrode’s
buffer. For use in the aggregation assay, the suspension was
then made up to 1 mM CaCl2 and supplemented with indo-
methacin (10 μg/ml; 28 μM). Platelets to be used for the calcium
signaling experiments were obtained as described above and
harvested by centrifugation from the platelet-rich plasma frac-
tion obtained immediately after the first centrifugation step
used to process the citrate-anticoagulated blood. The platelet
pellet obtained from the platelet-rich plasma was resuspended
in the isotonic Fluo-3-containing HEPES buffer, pH 7.4, described above, except that calcium was omitted from the
solution for the Fluo-3 uptake procedure and the two subse-
quent washes. For aggregation studies, the resulting washed
platelet suspension in the indomethacin-containing calcium-
replete buffer was allowed to stand at room temperature for 1 h
to allow for degradation of residual prostaglandin I2 before use
as 0.4-ml aliquots (2–3 × 10^{10} platelets/ml) in calcium-replete
(1 mM) Tyrode’s buffer, pH 7.4. Light transmission was moni-
tored with a dual channel aggregometer (Payton Scientific, Buf-
falo, NY). Agonists were added directly to the 0.4-ml suspen-
sion at 37 °C, and aggregation was quantified as a percentage (%
thrombin) of the maximal aggregation (i.e. maximal increase in
light transmission) caused by 1 unit/ml (10 nM) human plasma
thrombin (catalog number 605195, lot B37722, 3,186 NIH
units/mg; Calbiochem). For calculating the molar concen-
tration of thrombin, 1 unit/ml was taken as equivalent to 10 nM.

*Aorta Relaxation Assay—* The endothelium-intact rat and
murine aortic ring assay used to monitor the responses to the
PAR-activating peptides and the kallikreins was essentially the
same as that described previously for measuring the actions of
PAR2-activating peptides (46, 47). In brief, male Sprague-Daw-
ley rats (250–300 g) or male C57/Bl6 mice (either wild-type or
PAR2 null, about 50–70 g), cared for in accordance with the
Guidelines of the Canadian Council on Animal Care, were
killed by cervical dislocation. Clot-free portions of aorta were
dissected free of adherent connective tissue, and endothelium-
intact rings (approximately 2 mm length × 2 mm outer diam-
eter) were cut for use in the bioassay. Aortic rings were equili-
brated at 1 g resting tension for 1 h at 37 °C in a gassed (5% CO2,
95% O2) modified Krebs-Henseleit buffer, pH 7.4, of the follow-
ing composition: NaCl (118 mM), KCl (4.7 mM), CaCl$_2$ (2.5 mM), MgCl$_2$ (1.2 mM), NaHCO$_3$ (25 mM), KH$_2$PO$_4$ (1.2 mM), and glucose (10 mM). The relaxant actions of trypsin, the hK$_5$s, or the PAR-activating peptides (about 2 nM SLIGRL-NH$_2$ or 5 nM TFLLR-NH$_2$) were measured in endothelial intact aortic rings that were pre-contracted with 100 nM phenylephrine. A relaxant response to 10 nM acetylcholine (a 60–95% reduction of the tension generated by 100 nM phenylephrine) was taken as a positive index for an intact endothelium. To assess the contribution of the endothelium to the relaxation response, endothelium-free preparations were used, in which the endothelium was destroyed by rolling the aortic rings against a thin wire. The absence of endothelium was verified by observing an absence of a relaxant response to 10 nM acetylcholine. Peptides were added directly to the organ bath (4-ml cuvette), and the development of tension and subsequent relaxation exhibited by the rings was monitored using either Grass or Statham force-displacement transducers. When present, L-NAME (0.1 mM) was added to the organ bath 10 min prior to the addition of other reagents.

RESULTS
hK-mediated Proteolysis of Synthetic Tethered Ligand Peptide Sequences

Analysis by HPLC Followed by Mass Spectral Analysis—Because our study dealt with kallikreins of human origin, we elected first to evaluate their ability to cleave the peptides corresponding to regions of the N-terminal domains of human PAR$_1$, PAR$_2$, and PAR$_4$, with a focus on hK14 as a prototype kallikrein (Table 2) and PAR$_2$ as its main target. Cleavage by hK5 and -6 was also studied, but the limited amounts of these enzymes available for our study did not permit an extensive analysis as with hK14. HPLC separation followed by mass spectral identification of the proteolysis fragments in the peaks generated by hK14 from each of the synthetic tethered ligand sequences of hPAR$_1$, PAR$_2$, and PAR$_4$ (Fig. 1) showed that the expected receptor-activating sequences (SFLLRN... for PAR$_1$; SLIGKV... for PAR$_2$; and GYPGQV... for PAR$_4$) were released by cleavage at the R/S (PAR$_1$ and PAR$_2$) or R/G (PAR$_4$) bonds (Table 2 and Fig. 1). The degree of peptide cleavage demonstrated by the relative peak areas of the HPLC analysis indicated that although there was significant cleavage of the hPAR$_2$- and hPAR$_4$-derived peptides (>50%), only a modest cleavage (about 20%) of the hPAR$_1$ peptide had occurred (Fig. 1, tracings A–C). The HPLC analysis also demonstrated appreciable hK14 cleavage of the human PAR$_2$ sequence at the arginine upstream of the tethered ligand sequence (TNR/SSK). In addition, identification of the PAR$_2$ cleavage sequence, NATLDPRSFLLR (Fig. 1, tracing A), indicated that hK14 cleaved within the tethered ligand sequence of hPAR$_2$ at a site (LLR/NPN) that would “disarm” the receptor. This cleavage would remove the tethered ligand sequence from the remainder of the receptor, thereby preventing its activation by thrombin. HPLC-mass spectral analysis also demonstrated hK14 cleavage of the comparable rat PAR$_2$ and PAR$_4$, N-terminal peptide sequences at the R/S and R/G sites that would generate the receptor-activating sequences (Fig. 1, tracings D and E, and Table 2). The time course of hK14 cleavage of the human and rat PAR$_2$ peptides revealed that at 30 min, the receptor-activating peptides (SLIGKV... or SLIGRL...) represented the
major proteolysis products generated from both peptides. For the human PAR₂ sequence, this peptide (SLIGKV...) represented 14% of the amount of the intact peptide at 5 min, 39% at 10 min, and 120% of the remaining amount of intact peptide at 30 min (Fig. 1, tracing B). The rate of hK14 hydrolysis of the rat PAR₂ peptide was more rapid, with the amount of receptor-activating peptide released at 30 min (SLIGKV...) representing about 250% of the remaining intact peptide (Fig. 1, tracing D). Thus, the key R/S bond that reveals the receptor-activating sequence represents the main hK14 target in both human and rat PAR₂. These results paralleled closely the cleavage products yielded by trypsin-mediated hydrolysis of the PAR-derived peptides (not shown). In this regard, the cleavage by trypsin to yield the human and rat PAR-activating peptide sequences was greater in extent than that of hK14.

A comparable HPLC-mass spectral analysis of the cleavage products generated by hK5 and -6 from the PAR-derived peptides yielded results consistent with those shown for hK14 in Fig. 1, with the exception of the hydrolysis of the PAR₄ peptides. Peptide hydrolysis by these two hKs released significant amounts of the receptor-activating peptide for human and rat PAR₂ and small amounts of the hPAR₁-activating peptide (not shown). A peptide suggesting a disarming cleavage of PAR₁ (Fig. 1, tracing A, 3rd peak on the right) was not observed for the cleavage of the hPAR₂ peptide by either hK5 or hK6. The human PAR₂ peptide was cleaved by both hK5 and hK6 to yield a receptor-activating peptide (about 10% of the uncleaved peptide at 30 min), but no release by hK5 and -6 of a PAR₂-activating peptide was detected by HPLC analysis from the rat-derived PAR₂ sequence, despite its detection by the LC/MS approach (not shown and see below). In summary, we found that like hK14, both hK5 and hK6 could cleave the human PAR₁, PAR₂, and PAR₄ synthetic peptide sequences to generate receptor-activating peptides, with a predominant cleavage of the hPAR₂ peptide compared with PAR₁ and PAR₄. Although hK5 and hK6 cleavage of the rat PAR₂-derived sequence also yielded significant amounts of a receptor-activating peptide, the rPAR₂ sequence did not yield amounts of a receptor-activating peptide that could be detected by HPLC analysis.

**Analysis of Peptide Proteolysis by LC-MS or MALDI**—In addition to the approach described above, we subjected the entire proteolysis reaction mixture to LC-MS and MALDI analysis without prior HPLC separation of the cleaved peptides. This approach enabled us to detect minor cleavages that would have been missed by the HPLC separation approach but did not permit an analysis of the relative abundance of the cleavage products identified. Cleavage of rat PAR₂ was not studied. This analysis (Table 2) confirmed the release by hK5, -6, and -14 of the tethered ligand sequences from human PAR₁, PAR₂, and PAR₄ and from rat PAR₂. A minor cleavage of rat PAR₂ to yield its receptor-activating sequence was also suggested (Table 2). In addition, the analysis confirmed the cleavage of the hPAR₁ sequence at the LLR/NP bond that would disarm the receptor. Other cleavages in the PAR peptides were also detected at predicted tryptic sites of serine proteinase proteolysis (at a lysine either within or C-terminal to the tethered ligand sequence of human PAR₂ or at a lysine C-terminal to the tethered ligand of rat PAR₂, to yield the peptide sequence LNESKSPDKPNPRG-FPGK). This second cleavage would not be expected, given the inability of trypsin to cleave if a proline residue is on the carboxyl side of the cleavage site, because of the proline amino group. Although these cleavages found by the LC-MS or MALDI approaches must be presumed to result from minor sites of cleavage (the majority of them were not detected by the HPLC analysis; see above), they would in principle also disarm the two PARs. In general, cleavage of the PAR peptide sequences by the hKs reflected comparable cleavages caused by trypsin, especially in terms of revealing the tethered ligand sequences of the PARs. Added to the expected cleavages at arginines or lysines, the LC/MS and MALDI analyses pointed to unlikely chymotryptic sites of cleavage caused by hK14, e.g. at the SF/LL bond of human PAR₁, or the GF/YPG site in hPAR₄ (Table 2). These cleavages may be due to the recognized chymotryptic activity of hK14 (48). Other unusual sites of cleavage were also suggested by the LC/MS and MALDI analyses. However, the expected chymotryptic and other peptides were not detected upon HPLC analysis (above).

Thus, hK14, -5, and -6 can cleave synthetic peptides representing the N-terminal sequences of PAR₁, PAR₂, and PAR₄ to expose their receptor-activating moieties. These sequences, when unmasked by the hKs in vivo, would lead to activation of the receptors in intact cells. In addition, these three proteinases can also cleave at sites that would excise the tethered ligand sequences, thereby disarming these PARs. The major sites of hK cleavage shown by the HPLC peaks in Fig. 1 as well as other minor cleavages detected by the LC-MS and MALDI analyses are summarized in Table 2, where both the major (bold upward arrows, detected by HPLC-MS analysis) and minor sites (downward arrows, detected by LC-MS analysis) of cleavage are indicated. A possibility that cannot be ruled out is that the unexpected cleavage products detected by the LC-MS/MALDI analysis of the entire hydrolysis mixture may have resulted from peptide fragmentation because of the mass spectral procedure itself or, although unlikely, because of the presence of undetected non-hK protease contaminants in the hK preparations. The potential physiological impact of the minor sites of cleavage is difficult to assess.

**Evaluation of hK-mediated PAR₂ Cleavage in Intact Cells**

We have found previously that the observed cleavage of model PAR peptides by trypsin or thrombin may or may not reflect the ability of the enzymes to cleave/activate or disarm/inhibit the receptors in intact cells (35). Thus, to assess whether hK14 can cleave intact PAR₂ at the cell surface, we used an immunohistochemical approach to monitor PAR₂ cleavage in intact rat PAR₂-expressing KNKR cells (KNKRPAR₂), as outlined under “Materials and Methods.” The expressed rat receptor was used for our study because of our previous success with the analysis of trypsin cleavage of this receptor in intact cells and intact tissues (35, 40). The analysis depends upon the reactivity of two target antisera (B5 and SLAW-A) with two distinct domains of rat PAR₂ (35). The B5 antiserum detects the cleavage-activation sequence of PAR₂, whereas the SLAW-A antiserum detects only the pre-cleavage sequence. When KNKR PAR₂ cells were incubated with hK14 under conditions that would
result in calcium signaling (see below), morphometric analysis of the immunoreactive cells revealed that there was a loss of reactivity with the SLAW-A antisera (a reduction in signal relative to untreated cells of 80 ± 3%, mean ± S.E., n = 5) but retention of reactivity toward B5 (Fig. 2). Trypsin also removed the SLAW-A epitope (reduction of SLAW-A fluorescence signal by 84 ± 2% relative to control cells) (Fig. 2). These data indicated that cleavage at the GR/SL bond of PAR2 had occurred, removing the epitope(s) reacting with the SLAW-A antisera, but cleavage at other downstream sites (either trypic or chymotryptic), which would have released the epitope(s) reacting with the B5 antiserum, had evidently not occurred. Treatment of the KNRKPAR2 cells with hK6 produced results comparable with those obtained with hK14 (a reduction in the SLAW-A signal by 69 ± 5%, relative to control cells). hK5 was also able to remove the epitope(s) detected by the SLAW-A antisera but to a lower degree (a reduction in signal of 28 ± 4%, mean ± S.E., n = 5) than was observed for hK6 and -14 (Fig. 2). As was evident from examining multiple microscopic fields like those shown in Fig. 2, neither hK6 nor hK5 was able to decrease the reactivity of the B5 antisem with the cells. The results indicated that, as for hK14, the tethered ligand had been proteolytically revealed but that appreciable cleavage downstream of the tethered ligand sequence had not occurred.

**Calcium Signaling Triggered by hK-mediated Activation of PAR2 and PAR2**

To determine whether hK14 cleaves and activates PAR2, we measured its effects on calcium signaling by both the human and rat receptors expressed in intact cells. We first tested KNRKPAR2 cells that were used for the immunohistochemical monitoring of PAR2 activation. These cells express only rat PAR2 and not the other PARs. In the KNRKPAR2 cells, hK5, -6, and -14 were all able to generate a calcium signal comparable with that of trypsin (Fig. 3A, tracing A) but did not produce a signal in the nontransfected KNRK cells at the same enzyme concentrations (not shown). Furthermore, pre-desensitizing the PAR2-generated calcium signal in the KNRKPAR2 cells by pre-exposure to a high concentration of the PAR agonist, SLIGRL-NH2 (50 μM), prevented the subsequent kallikrein-triggered calcium signal (Fig. 3A, tracing B). Finally, in the KNRK cell line expressing the rat PAR2(R36A) mutant receptor, in which the R36A mutation abrogates trypsin-mediated (but not SLIGRL-NH2-triggered) activation of PAR2 (Fig. 3A, tracing C, first result on the left) (35), none of kallikreins 5, 6, or 14 caused a calcium signal (Fig. 3A, tracing C, right-hand results) at concentrations that did so in the wild-type KNRKPAR2 cells (Fig. 3A, tracing A, right-hand results). As expected, the selective PAR2-activating peptide, SLIGRL-NH2, did elicit a calcium signal in the PAR2(R36A) cells (Fig. 3A, tracing C, right-hand tracings). Thus, kallikreins 5, 6, and 14 activate rat PAR2 at the same Arg36/Ser37 site as trypsin to expose the tethered ligand sequence. The concentration-effect curve for hK14-mediated activation of PAR2 calcium signaling was compared with the curves for hK6 and trypsin in the KNRKPAR2 cells (Fig. 3B). The relative potencies (inverse of the EC50 values) of the three enzymes were as follows: trypsin > hK6 > hK14, with the potency of hK14 about 8 times less than that of trypsin. Of note is that although hK6 exhibited a lower EC50 than that of hK14, indicating a higher potency for PAR2 activation, the maximum calcium signal generated by hK14 was much greater than that for hK6 (Fig. 3B), suggesting a complex action of hK6, comprising both receptor activation (lower hK6 concentrations) and inactivation (higher hK6 concentrations).

We next examined the ability of hK5, -6, and -14 to activate calcium signaling via human PARs in the HEK cell line (Fig. 4, A and B). In contrast with the KNRKPAR2 cell line that expresses a high abundance of only one of the PARs, the human HEK cells
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constitutively express both PAR_1 and PAR_2 (but not PAR_3) at levels one might expect in vivo, so the relative selectivity of the kallikreins to activate either PAR_1 or PAR_2 (or both) could be evaluated. All three kallikreins caused a comparable elevation of intracellular calcium in the HEK cells, presumably from the combined activation of PAR_1 and PAR_2 (Fig. 4A, tracing A). The ability of hK14 to activate either of PAR_1 or PAR_2 individually was assessed using a receptor desensitization assay (Fig. 4A, tracings B–D). In the desensitization assay, the calcium signal caused by hK14 in the HEK cells after first desensitizing PAR_1 with a high concentration (100 μM) of the selective PAR_1-activating peptide, TFLLR-NH_2, indicated an activation of PAR_2 (Fig. 4A, tracing B). Conversely, the calcium signal generated by hK14 in HEK cells, first desensitized by a saturating concentration of the selective PAR_2-activating peptide, SLIGRL-NH_2 (100 μM), demonstrated an activation of PAR_1 (Fig. 4A, tracing C). Finally, first pre-desensitizing the HEK cells with a high concentration (200 μM) of the nonselective PAR-activating peptide, SFLLR-NH_2, which affects both PAR_1 and PAR_2, eliminated the hK14-induced signal entirely, demonstrating that the hK14-triggered HEK calcium signals did not arise from the activation of receptors other than PAR_1 and PAR_2 (Fig. 4A, tracing D). From the peak heights of the calcium signals observed after pre-desensitization of either PAR_1 (PAR_2 signal seen in Fig. 4A, tracing B) or PAR_2 (a much smaller PAR_1 signal seen in Fig. 4A, tracing C), relative to the integrated signals caused in the cells prior to desensitization (i.e. generated by simultaneous activation of both PARs; see right-hand tracings in Fig. 4A, results B and C), it was possible to deduce that the integrated signal caused by hK14 acting on both PAR_1 and PAR_2 in the nondesensitized cells came largely from PAR_2 activation. With this same approach, by first desensitizing PAR_1 with TFLLR-NH_2, it was possible to determine the concentration-effect curve for the selective activation of human PAR_2 by hK14 (EC_{50} = 8 units/ml; middle curve in Fig. 4B); and by pre-desensitizing with SLIGRL-NH_2, the concentration-effect curve for hK14 activation of human PAR_1 was determined (EC_{50} = 10 units/ml; bottom curve in Fig. 4B). As shown in Fig. 4B, the potency of hK14 for activating a calcium signal via PAR_2 (middle curve in Fig. 4B) was close to its potency for signaling via PAR_1 (lower curve, Fig. 4B), given the equivalent EC_{50} values of 8–10 units/ml. However, the maximum calcium signal caused by the selective activation of PAR_2 by hK14 at 20 units/ml was equivalent to the maximum signal caused by the selective activation of PAR_2 by a maximally active concentration (100 μM) of the PAR-activating peptide, SLIGRL-NH_2 (compare the calcium signals shown in the left-hand results of Fig. 4A, tracings A

FIGURE 3. A, mobilization of intracellular calcium by hKs, trypsin, and SLIGRL-NH_2 in KNRKrPAR_2 and KNRKrPAR_2(R36A) cells. Tracing A, representative calcium response data triggered by trypsin (Trp, 1 unit/ml; ○), hK14 (▴), hK6 (□), hK5 (■), and calcium ionophore (CI, △). Tracing B, desensitization of hK14 action by pre-activation of PAR_2 with SLIGRL-NH_2. Tracing C, activation of calcium signaling by SLIGRL-NH_2 but not by trypsin and hK14 in KNRKrPAR_2(R36A) cells. The cellular response to calcium ionophore (2 μM) is shown at the start of tracing A. An upward deflection (arrow on ordinate) indicates an increase in intracellular calcium. The scale for time and calcium signaling in arbitrary units (upward deflection in centimeters) is shown by the inset. On average the basal calcium concentration was 30 nM, and the ionophore-triggered peak calcium concentration was in the range of 340 nM. Data are representative of three or more replicate experiments done with independently grown cell cultures. B, concentration-effect curves for mobilizing intracellular calcium in KNRKrPAR_2 cells by hK14, hK6, and trypsin. The calcium signal, relative to that caused by 2 μM ionophore (% A23187), was monitored for increasing enzyme concentrations. Each data point represents the average ± S.E. (bars) for three or more measurements at each enzyme concentration, done with separately grown cell cultures. Error bars smaller than the size of the symbols are not shown.
and C). In contrast, the maximum calcium signal generated by the selective activation of PAR₁ by hK14 at its optimal concentration for causing a calcium signal (20 units/ml) was substantially lower than the maximum calcium signal generated by selectively activating PAR₁ with thrombin (top curve in Fig. 4B). The considerably lower maximum PAR₁-derived calcium signal caused by hK14 relative to that caused by thrombin (or, relative to the TFLLR-NH₂ peptide-stimulated calcium signal), pointed to a disarming/inhibition of PAR₁ by hK14. We thus tested the ability of pretreatment of HEK cells with hK14 to reduce the subsequent calcium signal caused by thrombin activation of HEK cell PAR₁. As shown in Fig. 5A, pretreatment of HEK cells with hK14 at a concentration that did not by itself desensitize the calcium signal caused by TFLLR-NH₂ (i.e. no desensitization of PAR₂) caused only a small signal (relative to that triggered by thrombin or TFLLR-NH₂) via PAR₁ activation.

**hK-mediated Disarming/Inhibition of HEK Cell PAR₁**

Proteolytic removal of the tethered ligand can disarm a PAR for activation by its selective proteinase. The much smaller maximal calcium signal generated by hK14 via selective PAR₁ activation, relative to the maximum PAR₁ signal caused by either thrombin or the PAR₁-activating peptide, TFLLR-NH₂, pointed to a disarming/inhibition of PAR₁ by hK14. We thus tested the ability of pretreatment of HEK cells with hK14 to desensitize both PAR₁ and PAR₂. Tracings are representative of three or more independently conducted experiments done with cells derived from independently grown cultures. The scale for time (min) and calcium signaling (upward deflection in centimeters) is shown by the inset. A, concentration-effect curves for hK14-mediated mobilization of intracellular calcium in HEK cells by selectively activating either PAR₁ (○) or PAR₂ (■); comparison with thrombin-mediated activation of PAR₁ (□); to monitor hK14 signaling selectively either via PAR₁ or via PAR₂, HEK cells were first desensitized by either the selective PAR₁ agonist, SLIGRL-NH₂ (revealing signaling by PAR₁; □, bottom curve) or by the selective PAR₂ agonist, TFLLR-NH₂ (revealing activation by PAR₂, ○, middle curve). Calcium signaling by increasing concentrations of hK14 in the pre-desensitized cells was monitored. The signaling of hK14 via PAR₁ (bottom curve) can be compared with signaling by thrombin via PAR₁ (□, top curve). In the HEK cells, thrombin can signal only via PAR₁, because it is unable to activate PAR₂. Data points represent the average values (± S.E.; bars) at each enzyme concentration, obtained for three or more measurements with independently grown cell cultures. Error bars smaller in magnitude than the symbols are not shown.
right-hand bar), reduced the subsequent signal caused by thrombin activation of PAR1 (Fig. 5A, tracing A, and lower panel, left-hand bar). It is important to note in interpreting this experiment using the HEK cells expressing both PAR1 and PAR2, that unlike trypsin or hK14, thrombin is not able to activate a calcium signal in the HEK cells via PAR2. The concentration-effect curve for the ability of hK14 to disarm the ability of thrombin to activate HEK cell PAR1 is shown in Fig. 5B. At a concentration of 5 units/ml, hK14 was able to reduce the thrombin-mediated signal (i.e. disarming/inhibition) by more than 50%, without desensitizing the cell response to TFLLR-NH2. If at this concentration of hK14 (5 units/ml) the cells were first exposed to the enzyme for 10 min at room temperature and the hK14 was then removed by washing the cells by centrifugation and re-suspension prior to the calcium flux measurement, the thrombin response was also markedly diminished, but the response to TFLLR-NH2 was retained (not shown). This result indicated a disarming of PAR1, rather than an inhibition of thrombin action either by thrombin proteolysis or by a tight noncatalytic binding of hK14 to the cleavage activation site that would prevent thrombin access. The EC50 value for the ability of hK14 to disarm/inhibit PAR1 (about 4.5 units/ml, left-hand upper curve in Fig. 5B) was lower than its EC50 value (about 10 units/ml, right-hand, upper curve in Fig. 5B) for causing a desensitization of the selective activation of PAR1 in the same cells by TFLLR-NH2. Thus, in cells co-expressing PAR1 and PAR2, hK14, and presumably the other hKs, can have complex actions, activating PAR2 and, depending on the concentration, either disarming/inhibiting or activating PAR1.

**hK-mediated Activation of PAR4**

**Rat Platelet Aggregation Assay**—Because rat platelets possess PAR4, but neither PAR1 nor PAR2, thrombin-mediated aggregation can be attributed to PAR4 activation and not to the activation of other PARs (49). We therefore used a rat platelet aggregation assay to evaluate the ability of hKs to activate rat PAR4. Like thrombin and trypsin that act via PAR4 in this preparation, hK14 caused aggregation. Surprisingly, however, neither hK5 nor hK6 were able to do so at concentrations that could activate PAR2 (20 units/ml: Fig. 6). The concentration-effect curve for hK14-mediated rat platelet aggregation (EC50 = 14 units/ml) showed that its potency was about 2-fold lower than that of trypsin (EC50 = 7 units/ml) for activating rat platelet PAR4 (Fig. 6, left-hand curve).

**hK-mediated Activation of Rat PAR4 Expressed in HEK Cells**—To assess further the ability of hK14 to activate rat PAR4, we used HEK cells expressing recombinant PAR4 in addition to the constitutive expression of human PAR1 and PAR2. Activ-
tion of PAR4 with the PAR4-selective activating peptide, AYPGKF-NH2 (which cannot activate either PAR1 or PAR2), caused an increase in intracellular calcium (Fig. 7A, tracing B) in cells that had already been desensitized with SFLLR-NH2 to eliminate any signaling by either PAR1 or PAR2. In cells that were first desensitized with SFLLR-NH2, which simultaneously silences signaling by PAR1 and PAR2, hK14 yielded a robust calcium signal (Fig. 7A, tracing A). In the same type of assay, cells pre-desensitized by the combined agonists of PAR1, PAR2, and PAR4 (SFLLR-NH2 plus AYPGKF-NH2) no longer yielded a calcium signal in response to hK14 (Fig. 7A, tracing B, right-hand portion). Furthermore, HEK cells that were not transfected with the rat PAR4 construct, upon being desensitized with SFLLR-NH2 (to silence both PAR1 and PAR2), no longer responded to hK14 (data not shown and result shown in Fig. 4A, tracing D). Thus, unequivocally, hK14 was able to activate PAR4 in the HEK-rPAR4 cells and did not cause its calcium signal via a non-PAR mechanism. To evaluate the sensitivity of rat PAR4 to activation by hK14, HEK-rPAR4 cells were PAR1/PAR2 pre-desensitized by treatment with a relatively high concentration of SFLLR-NH2 and were then exposed to increasing concentrations of hK14 (Fig. 7B, right-hand curve). The potency of hK14 in this assay (EC50 ≈ 15 units/ml) (Fig. 7B, right-hand curve) was in the same range as that of trypsin (EC50 ≈ 9 units/ml) (Fig. 7B, left-hand curve). These results for hK14-mediated activation of rat PAR4 expressed in HEK cells were entirely in keeping with the ability of hK14 to activate PAR4-dependent rat platelet aggregation (above) and to release a PAR4-acti-
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In the human platelet, which expresses PAR1 and PAR4 in the same platelet aliquot. In the human platelet, which does not possess PAR2, aggregation is triggered by the combined action of PAR1 and PAR4 (4). There was therefore a differential action of the three kallikreins in the rat platelet preparation (Fig. 6). This result indicated that in the human platelet preparation, the main calcium signal generated by hK14 was triggered by PAR4. In keeping with this result, treatment of the platelets with a PAR4-selective desensitizing concentration of AYPGKF-NH2 substantially reduced the hK14-triggered signal, revealing only a very small calcium response (Fig. 8, tracing C). As already alluded to, this small residual response in the presence of AYPGKF-NH2 was presumably because of PAR4 activation. Finally, the combined treatment of the platelets with the PAR antagonist SCH 79797 in combination with a desensitizing concentration of AYPGKF-NH2 eliminated the hK14-triggered calcium signal as well as the signal generated by either thrombin or TFLLR-NH2 (Fig. 8, tracing D). This result indicated that hK14 was acting via the PAR4s and not via another receptor. In contrast with the ability of PAR4 desensitization to reduce the hK14 signal by about 90% (Fig. 8, tracing C), the human platelet signal generated by thrombin after PAR4 desensitization, because of PAR4 activation, was reduced by only about 20% (Fig. 8, tracing E). Thus, in contrast with the thrombin-triggered signal generated largely by PAR4 activation, calcium signaling in human platelets driven by hK14 appeared to result primarily (about 90%) from activation of PAR4 and not PAR1 (compare tracings C and E in Fig. 8).

In contrast with hK14, neither hK5 nor hK6 (20 units/ml) caused a calcium signal in the human platelets, indicating a lack of activation of either of PAR1 or PAR4. Furthermore, hK5 or hK6 did not trigger human platelet aggregation, whereas hK14 did (not shown). These results paralleled the lack of action of these two kallikreins in the rat platelet preparation (Fig. 6). There was therefore a differential action of the three kallikreins in terms of activating human (and rat) PAR4.

In sum, the calcium signaling experiments showed that in human platelets, as in the rPAR4-expressing HEK cells (Fig. 7A), hK14 can preferentially activate PAR4 rather than PAR1, which is in contrast disarmed/inhibited by hK14. This selectivity of hK14 for PAR4 over PAR1, in the platelet is the opposite of the selectivity observed for thrombin, which preferentially activates human platelet PAR1, although also able to activate PAR4 at higher concentrations (4).

Vascular Bioassays

Aorta Ring Relaxation Assays Using Rat and Murine Vascular Tissue—Activation of either PAR1 or PAR4 (46, 52) but not PAR4 (49) induces an endothelium-dependent, nitric oxide-mediated relaxation of rat or mouse aorta. To determine whether hKs could activate PARs in intact tissues, which unlike

vating sequence from the synthetic rat PAR4-derived tethered ligand peptide (Table 2).

hK-mediated Activation of PAR4 in Human Platelets—To assess the ability of hK14 to activate human PAR4, we used a human platelet calcium signaling assay (50). As opposed to an aggregation assay, the calcium signaling assay permits a sequential and concurrent evaluation of the activation of platelet PAR1 and PAR4 in the same platelet aliquot. In the human platelet, which does not possess PAR2, aggregation is triggered by the combined action of PAR1 and PAR4 (4). That said, activation of PAR1 and PAR4 can have distinct effects on platelet function (51), and we therefore wished to determine whether hK14 might differentially activate PAR1 and PAR4 in the human platelet preparation. As shown in Fig. 8, the receptor-selective activating peptides for PAR1 (TFLLR-NH2, Fig. 8, tracing A) and PAR4 (AYPGKF-NH2, Fig. 8, tracing C) each generated a strong calcium signal in the human platelet preparation, as did hK14 (20 units/ml, right-hand portions of results in Fig. 8, tracings A–C). When the human platelets were first treated with the PAR1 antagonist SCH 79797, to eliminate either TFLLR-NH2 or thrombin signaling via PAR1 (Fig. 8, left portion of tracing B, and data not shown for thrombin), or with a PAR4-desensitizing concentration of TFLLR-NH2 (Fig. 8, tracing A), hK14 (20 units/ml) still caused a calcium signaling response that was almost the same as that observed in nondesensitized cells (Fig. 8, tracings A and B; compare the responses on the far right with the responses to hK14 in antagonist-treated or PAR4-desensitized cells in the left-hand tracings in each figure). This result indicated that in the human platelet preparation, the main calcium signal generated by hK14 was triggered by PAR4. In keeping with this result, treatment of the platelets with a PAR4-desensitizing concentration of AYPGKF-NH2 substantially reduced the hK14-triggered signal, revealing only a very small calcium response (Fig. 8, tracing C). As already alluded to, this small residual response in the presence of AYPGKF-NH2 was presumably because of PAR4 activation. Finally, the combined treatment of the platelets with the PAR antagonist SCH 79797 in combination with a desensitizing concentration of AYPGKF-NH2 eliminated the hK14-triggered calcium signal as well as the signal generated by either thrombin or TFLLR-NH2 (Fig. 8, tracing D). This result indicated that hK14 was acting via the PARs and not via another receptor. In contrast with the ability of PAR4 desensitization to reduce the hK14 signal by about 90% (Fig. 8, tracing C), the human platelet signal generated by thrombin after PAR4 desensitization, because of PAR1 activation, was reduced by only about 20% (Fig. 8, tracing E). Thus, in contrast with the thrombin-triggered signal generated largely by PAR4 activation, calcium signaling in human platelets driven by hK14 appeared to result primarily (about 90%) from activation of PAR4 and not PAR1 (compare tracings C and E in Fig. 8).

In contrast with hK14, neither hK5 nor hK6 (20 units/ml) caused a calcium signal in the human platelets, indicating a lack of activation of either of PAR1 or PAR4. Furthermore, hK5 or hK6 did not trigger human platelet aggregation, whereas hK14 did (not shown). These results paralleled the lack of action of these two kallikreins in the rat platelet preparation (Fig. 6). There was therefore a differential action of the three kallikreins in terms of activating human (and rat) PAR4.

In sum, the calcium signaling experiments showed that in human platelets, as in the rPAR4-expressing HEK cells (Fig. 7A), hK14 can preferentially activate PAR4 rather than PAR1, which is in contrast disarmed/inhibited by hK14. This selectivity of hK14 for PAR4 over PAR1, in the platelet is the opposite of the selectivity observed for thrombin, which preferentially activates human platelet PAR1, although also able to activate PAR4 at higher concentrations (4).
the cells may contain extracellular proteinase inhibitors in vivo, we examined the actions of the hKs in rat and murine aorta preparations. hK5, -6, and -14 caused a relaxation of endothelium-intact rat aorta tissue that had been preconstricted with phenylephrine (Fig. 9A, tracing A). As with our previous results for trypsin-mediated PAR-triggered vasorelaxation, the relaxant response caused by hK14 was absent in endothelium-free preparations (not shown) and was abolished by the nitric-oxide synthase inhibitor L-NAME (Fig. 9A, tracing B). hK5 and -6 also caused an endothelium-dependent relaxation that was abolished by L-NAME (not shown).

To explore the mechanism of hK14-induced relaxation further, we evaluated its ability to relax phenylephrine pre-constricted aorta from wild-type mice (Fig. 9A, tracing C) and PAR2 null mice (Fig. 9A, tracing D). The concentration-effect curve for this relaxant action of hK14 in the wild-type murine aorta preparation was also determined (Fig. 9B), to ensure that the PAR null tissues had been tested at appropriately high concentrations of hK14. In the endothelium-intact murine aorta preparation, hK14 caused a relaxation response that was equivalent to that of acetylcholine (Fig. 9A, tracing C). The potency of hK14 for causing a relaxant response in the murine aorta preparation was close to that of trypsin (EC50 = 0.5 units/ml; Fig. 9B). Concentrations of hK14 toward the plateau of the concentration-effect curve (3 units/ml) failed to cause relaxation in pre-constricted tissues from the PAR2 null mice, which otherwise relaxed in response to PAR1 activation by TFLLR-NH2 or thrombin (Fig. 9A, tracing D). Thus, hK14 relaxed the mouse aorta by the selective activation of PAR2 and not via PAR1.

DISCUSSION

Kallikreins Can Affect Signaling by Either Activating or Disarming/Inhibiting PARs—The main finding of our study was that signaling via human, rat, and murine PARs can be regulated by human kallikreins, with hK14 targeting the activation of PAR2 and PAR4 and the disarming/inhibition of PAR1. We established unequivocally the ability of the hKs to regulate PAR activation through a number of independent biochemical, cell biological, pharmacological, and genetic approaches as follows: 1) HPLC and mass spectral analysis of the cleavage products yielded upon incubation of hK5, -6, and -14 with PAR N-terminal peptide sequences based on the cleavage/activation motifs.
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of PAR1, PAR2, and PAR4; 2) PAR-dependent calcium signaling responses in cells expressing PAR1, PAR2, and PAR4, and in human platelets; 3) a vascular ring vasorelaxation assay using rat and murine tissue as well as murine tissue from PAR2 null mice; and 4) a PAR4-dependent rat and human platelet aggregation assay. The actions of the hKs are in marked contrast to the action of plasma kallikrein (a genetically distinct enzyme) that is reported not to activate PAR2 (53). Given the conservation between the genetic loci of the kallikreins 4–15 in non-human species like the mouse (54), we suggest that non-human kallikreins that are orthologues of hK5, -6, and -14 would also be able to signal via the PARs with a potency comparable with that of pancreatic trypsin. To generalize our findings for a variety of species, we validated the ability of the hKs to affect PARs of human, rat, and murine origin, presuming that, just as is the case for human thrombin and trypsin, PARs from other species although not identical in sequence would nonetheless also be susceptible to kallikrein activation. Based on our findings we suggest that PARs from many species would be susceptible to kallikrein regulation. It can be anticipated that, as is the case for signaling via PARs by thrombin and trypsin, the activation by the hKs of calcium signaling via Gq also implies a potential activation of Gq and other downstream signaling events, like the stimulation of mitogen-activated protein kinase, known to be regulated via PAR1 and PAR2.

Our focus was principally on PAR2, known to play an important role in inflammation and nociception (18, 55), and on hK14, known to be elevated in the serum of individuals with ovarian or breast carcinoma (26). In a number of respects, the actions of hK5 and -6 paralleled those of hK14, particularly in terms of activating PAR2. However, the actions of hK5 and -6 were distinct from those of hK14 in terms of their inability to activate PAR4. The lack of activation of PAR4 by these two kallikreins is in keeping with the very minor cleavage by hK5 and -6 observed by HPLC analysis for the PAR4-derived peptides and the lack of hydrolysis by hK6 of a model PAR4 peptide described in work that appeared after completion of our study (56). We therefore anticipate that the other human kallikreins apart from hK5, -6, and -14 as well as kallikreins from other species will have both common and distinct actions via the different PARs.

Kallikreins as Potential Regulators of PARs in Vivo—Like the other kallikreins, hK14 is widely distributed in the body (26, 57), representing a prime candidate as a physiological regulator of PAR2 in many settings, adding to the ability of other serine proteinases like trypsin, factor VIIa/Xa, and matriptase to activate PAR2. Unlike human mast cell tryptase, another potential physiological activator of PAR2, hK14 was not restricted in its ability to activate the receptor by the glycosylation site in proximity to the cleavage-activation target of the enzyme (39, 58, 59). Thus, in contrast with trypsin (58), hK14 was able to reduce vascular tension via PAR2 activation in an intact rat and murine aorta tissue assay. That PAR2 and its R/S cleavage activation sequence were the targets for hK14 action was established as follows: 1) by the lack of cleavage/activation by hK14 of the R36A mutant of rat PAR2 in which the tryptic cleavage/activation site was changed to a bond not susceptible to tryptic cleavage (Fig. 3A, tracing C); 2) by the lack of a relaxation response of endothelium-intact aorta rings derived from PAR2 null mice that otherwise relaxed in response to PAR4 activation (Fig. 9A, tracings C and D); and 3) by the disappearance of the N-terminal peptide sequence released by tryptic cleavage of rat PAR2 (reduction of SLAW-A reactivity caused by hK14), with retention of the amino acid epitopes in the active tethered ligand sequence of rat PAR2 recognized by the B5 antibody (Fig. 2). In this respect, hK5 and -6 yielded results comparable with those found for activation of PAR2 by hK14. In the assay for PAR2 activation in the KNRKrPAR2 cells (Fig. 3B, calcium signaling), hK6 was less potent than trypsin but more potent than hK14. As already pointed out, however, the maximum response generated by hK6 (calcium signal) was lower than that of hK14. The lower maximum response to hK6 activation compared with hK14 could result from a competing increased rate of a disarming cleavage at higher hK6 concentrations.

Thus, in general, unlike pancreatic trypsin, with its somewhat limited tissue distribution, and unlike trypsin, with its restricted ability to activate PAR4, the widely distributed tissue kallikreins via PAR2 specifically and via PAR1, PAR2, and PAR4 in general would, in principle, be able to affect a large number of tissues in the body, ranging from platelets to the vasculature. This potential ability of kallikreins to regulate tissue function via the PARs can now be added to other proposed mechanisms (e.g. degradation of extracellular matrix, activation of growth factors or generation of bradykinin, and processing of growth factor-binding proteins) believed to explain the complex biological actions of these serine proteinases in a variety of pathophysiological settings (20). Thus, it will be important to evaluate in the future the potential inflammatory and nociceptive roles that the kallikreins may play via PAR4 activation in vivo. One key issue that remains is to establish whether the amounts of enzymatically active kallikreins are present in vivo at concentrations equivalent to the relatively high hK concentrations detected by immunnoassay. Such protein concentrations, if representative of active enzyme, would be sufficient to regulate the PARs.

Consequences of hK14 Disarming/Inhibition of PAR2—The HPLC analysis of the hK14 cleavage products of hPAR1 demonstrated a much lower degree of cleavage than for PAR2 and PAR4. Notwithstanding, hK14 hydrolysis of the PAR1 peptide yielded about equal amounts of peptides resulting from cleavage at sites that would either activate or disarm the receptor (Fig. 1A). In the HEK cell and human platelet test systems, it was clear that hK14 preferentially disarmed/inhibited PAR2 rather than causing activation. This result paralleled exactly our previous data obtained for the disarming of PAR3 by trypsin (36). The data demonstrating that the hK14–treated HEK cells, when washed free from the enzyme, were no longer sensitive to thrombin but responded well to the PAR1-activating peptide, TFLLR-NH2, pointed to a removal of the PAR1 tethered ligand, as predicted by the peptide proteolysis data. This preferential disarming/inhibition of PAR1 by hK14 is similar to the ability of neutrophil elastase to disarm/inhibit PAR2 (16, 17). For cell regulation, the enzymatic disarming/inhibition of a PAR may be as important as its activation. By preferentially activating human platelet PAR4 (Fig. 8) while disarming PAR1 (Figs. 5 and 8), kallikreins would promote the release of platelet endostatin and suppress the release of platelet vascular endothelial growth factor (51). The preferential activation of PAR4 versus PAR1
mirrors the action of plasmin on human platelets, wherein plasmin can both activate and disarm PAR4 (60, 61). Whether PAR4 would be activated or disarmed by hK14 (Fig. 5) would depend on the ambient concentrations of hK14 at a given location and on the relative rates of cleavage at the two trypsic cleavage sites. Furthermore, by selectively activating PAR2 and PAR4, while disarming PAR1, kallikreins in general and hK14 specifically could contribute to processes related to the inflammatory response (18, 49, 62). These PAR-targeted actions of the kallikreins would add to the known ability of at least one member of the family, hK1, to generate inflammatory/nociceptive bradykinin peptides from kininogen.

Our LC-MS and MALDI analysis of the complex PAR peptide proteolysis mixture generated by the hKs revealed unexpected nontrypsic cleavage products that we presume represented only minor constituents, because we did not detect the peptides upon HPLC analysis. These minor cleavage sites could also disarm the PARs. For instance, the cleavage of the hPAR1 peptide at the SF/LL bond by hK14 that would disarm PAR1 may reflect the minor chymotryptic activity that this kallikrein has been reported to exhibit (48). It is an open question as to whether these minor cleavages of the PAR sequences would play a role in regulating PAR activity in vivo.

Activation of PAR4—Human and rat PAR4 also served as a target for hK14-mediated activation but not for hK5 and -6. The ability of hK14 to activate PAR4 was demonstrated both by the rat platelet aggregation response to hK14, which is PAR4-dependent (49), and by the calcium signaling responses in rat PAR4-expressing HEK cells (Figs. 6 and 7). The results with the HEK cell calcium signaling assay showed that the potency of hK14 for activating rat PAR4 (EC50 ≈ 15 units/ml; Fig. 7B) was comparable with but lower than its potency for activating either rat or human PAR2 (EC50 ≈ 8 units/ml; Figs. 3B and 4B). In the human platelet calcium signaling assay, hK14 was also able to activate PAR4, as indicated by the calcium signal generated in the presence of a PAR1 antagonist and from the data obtained using the PAR desensitization protocol (Fig. 8). As already mentioned, neither hK5 nor hK6 was able to activate either rat or human PAR4, indicating differences between the hKs for targeting the PARs. Our data therefore suggest that distinct hKs apart from the ones we have studied may differentially affect PAR4.

Pathophysiological Implications of Kallikrein Signaling via PARs—The ability of kallikreins to signal via proteinase-activated receptors is of particular interest because of the wide distribution of kallikreins in many human tissues, their up-regulation by sex steroid hormones, and their increase in a variety of pathological conditions (20, 21). Thus, kallikreins may represent localized endogenous PAR modulators in a variety of settings, including cancer. In different tissues, kallikreins appear to be expressed in distinct groups, having similar enzymatic potencies and activities regulated by common tissue inhibitors or by other kallikreins of the same group. For example, kallikreins 6 and 8 are widely expressed in the brain (63) at sites of PAR2 and PAR4 expression (19), and may therefore serve as potential agonists of these receptors in the central nervous system in normal or pathological settings. Other possible pathophysiological roles for local kallikreins may be found in the skin, where PARs and kallikreins are co-expressed (7, 64, 65) either in normal or pathological settings such as psoriasis (66–68). In the skin, uncontrolled kallikrein action can lead to desquamation (69), a setting in which PARs may be inappropriately activated. Another situation in which kallikrein-mediated PAR activation may be important is in tumorigenesis, because there is now an extensive literature dealing with changes in both the PARs (7) and the kallikreins in the setting of cancer, including cancers of the ovary, breast, colon, pancreas, and prostate (21, 70). These changes in the kallikreins can be correlated with either a favorable or unfavorable prognosis (20, 21). As alluded to above, it will be essential in these settings to determine the proportion of the enzyme that is catalytically active. Previous studies done in vitro have identified many potential substrates for the kallikreins (e.g. kininogens, extracellular matrix, growth factor precursors, or binding proteins), many of which are related to tumor survival, metastasis, and angiogenesis (20, 21). One can now add the PARs to this list of potential hK substrates that in principle can affect tumor behavior. We suggest that kallikreins may have a dual role during cancer onset and progression, similar to the metalloproteinases (71). Like the hKs, the metalloproteinases can cleave proteins of the extracellular matrix (e.g. collagen, fibrinogen, and metalloproteinases) and activate receptors like the PARs (71), related to tumor growth, metastasis, and survival.

In summary, our data add the PARs to the list of tissue kallikrein targets that may explain the signaling properties that this enzyme family can have in many settings. Via the PARs, the kallikreins can potentially signal by a variety of G-protein-coupled signaling pathways (Gi, Gq, and G12/13), so as to regulate processes ranging from cell migration to tumor angiogenesis, growth, metastasis, and invasion. The effects that kallikreins may have in any situation will depend on the spectrum of kallikreins present, the levels of the active free enzyme, and the availability of PARs that may be expressed in any specific environment. In this regard, the kallikrein-PAR axis may be seen as a fruitful therapeutic target for a number of disease states.

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