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Proteinase-activated Receptors, Targets for Kallikrein Signaling*[S]

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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 PAR_1 and PAR_4, the endogenous enzymes responsible for activating PAR_2 in settings other than the gastrointestinal system, where trypsin can activate PAR_2, 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 PAR_1, PAR_2, and PAR_4; 2) PAR-dependent calcium signaling responses in cells expressing PAR_1, PAR_2, and PAR_4 and in human platelets; 3) a vascular ring vasorelaxation assay; and 4) a PAR_4-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 PAR_1, PAR_2, and PAR_4 and to disarm/inhibit PAR_1. Although hK5 and -6 were also able to activate PAR_2, they failed to cause PAR_4-dependent aggregation of rat and human platelets, although hK14 did. Furthermore, the relative potencies and maximum effects of hK14 and -6 to activate PAR_2-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 PAR_1, PAR_2, and PAR_4.

Proteinase-activated receptors (PAR_{1-4})[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, PAR_1, PAR_3, and PAR_4 are recognized physiological targets for thrombin (4), which does not efficiently activate PAR_2. How-

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[3] The abbreviations used are: PAR, proteinase-activated receptor; r, rat; h, human; hK, human kallikrein protein; KNRK, Kirsten virus-transformed normal rat kidney cells; KNRKrPAR$_2$, rat PAR$_2$-expressing KNRK cells; KNRKrPAR$_2$(R36A), KNRK cells expressing a trypsin-resistant rat PAR$_2$ mutant; L-NAME, *N*-ω-nitro-L-arginine methyl ester; Trp, trypsin; HPLC, high pressure liquid chromatography; MES, 4-morpholineethanesulfonic acid; AMC, amino-4-methylcoumarin; MALDI, matrix-assisted laser desorption ionization; LC-MS, liquid chromatography/mass spectrometry; PBS, phosphate-buffered saline.

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ever, PAR_2 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 PAR_2 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 PAR_2 *in vivo* (13–15). Although a number of other serine proteinases are known to trigger PAR_2 signaling (7), the endogenous enzymes responsible for activating PAR_2 in settings other than the gastrointestinal tract where trypsin can activate PAR_2 are unknown. Moreover, apart from the observations that elastase and chymase can disable PAR_2 (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 tryptic 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 PAR_1, PAR_2, and PAR_4; 2) PAR-dependent calcium signaling responses in cell lines expressing PAR_1, PAR_2, and PAR_4 and in isolated human platelets; 3) a vascular ring vasorelaxation assay using tissues from rats and both wild-type and PAR_2 null mice; and 4) a PAR_4-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 PAR_2 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 PAR_1 and PAR_4, and for comparison, we determined whether hK5 and -6 might also regulate PAR_1, PAR_2, and PAR_4. 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 PAR_1, PAR_2, and PAR_4.

TABLE 1
List of symbols

Agonist / Antagonist	Symbol used	Comment
Calcium Ionophore	I	Control calcium response
Trypsin	□	Standard tryptic proteinase
hK14 / hK14, PAR_2	◑	hK14 activity shown plus or minus pre-desensitization of PAR_1
hK6	◪	hK6 activity shown plus or minus desensitization of other PARs
hK5	■	hK5 activity shown plus or minus desensitization of other PARs
SLIGRL-NH_2	▽	Selective PAR_2 agonist
TFLLR-NH_2	▼	Selective PAR_1 agonist
SFLLR- NH_2	▲	Non-selective PAR_1/PAR_2 agonist, co-desensitizes both PARs 1 and 2
Thrombin, PAR_1	⊕	Activates PARs 1 and 4, but not PAR_2
hK14, PAR_1	◁	hK14 action in PAR_2-desensitized HEK cells
AYPGKF-NH_2	△	Selective PAR_4 agonist
SCH 79797	▶	PAR_1 antagonist
Phenylephrine	●	Contractile Alpha-1 adrenoceptor agonist
Acetylcholine	○	Endothelial muscarinic NO-releasing agonist
L-NAME	◀	Nitric Oxide Synthase Inhibitor

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^{ω}-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 PAR_1 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, SSPS, 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-Gln-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 abscissa 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 EC_{50} 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 PAR_1, PAR_2, and PAR_4 were selected for the proteolysis studies (critical tethered ligand-activating sequences are underlined) (Table 2): (*a*) $hPAR_1$, NATLDPRSFLLRNPNDKYE; (*b*) $hPAR_2$, *N*-acetyl-GTNRSSKGRSLIGKVDGTSHVTGKGVT-amide; (*c*) $hPAR_4$, GDDSTPSILPAPRGYPGQV; (*d*) $rPAR_2$, GPNSKGRSLIGRLDTP*YGGC* (non-PAR_2 residues, *YGGC*, added for affinity-column coupling and ^{125}I labeling); and (*e*) $rPAR_4$, LNESKSPDKPNPRGFPGKP-amide.

These peptides at a concentration of 100 μM were incubated with kallikreins 5 (3.1 units/ml), 6 (0.83 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 E_{214} 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).

Cell Culture and PAR-expressing Cell Lines

Kirsten virus-transformed normal rat kidney (KNRK) epithelial cell lines expressing either wild-type rat PAR_2 (KNRKr-PAR_2) or the trypsin-resistant rat PAR_2(R36A) variant were validated previously for studies of receptor activation (35) and were therefore used to evaluate the PAR_2-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 PAR_1 and PAR_2 were grown as for the KNRK cells, but in the absence of geneticin, as outlined for the PAR-mediated calcium signaling procedure described previously (36). A rat PAR_4-expressing HEK cell line HEKrPAR$_4$ was generated from a receptor clone kindly provided by Dr. W. A. Hoogerwerf, Galveston, TX (37). In brief, using the rat PAR_4 clone, an N-terminal Myc and C-terminal herpes simplex virus tag were added by PCR employing the primers: forward, 5′-GAGCAGAAGCTGATCAGCGAAGAGGACCTGCCGGCGGAATGCCAGACGCC-3′, and reverse, 5′-ATCTCGAGTCATCAGCAGTCCTCGGGGTCCTCGGGGGCCAGCTCGGGCTGCAGAAGTGTAGAGGAGCAAATG-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 mycrPAR4HSV. 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 assessed for expression by immunofluorescence. After selection, the rat PAR_4-expressing HEK cells (HEKrPAR$_4$) 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 PAR_2 Cleavage/Activation

To assess morphologically the ability of the hKs to cause the activation or "disarming" of PAR_2, we made use of a rat PAR_2 expression system (receptor expressed in the KNRK cells) along with two rat PAR_2-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 PAR_2 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 PAR_2 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-PAR_2 antisera (B5 and SLAW-A) were raised in rabbits and used as described elsewhere (12, 35, 39). The antibodies were generated against peptides that corresponded to sequences representing the potentially antigenic epitopes either in the pre-cleavage domain of wild-type rat PAR_2 (for SLAW-A, SLAWLLGGPNSKGR) or in the tethered ligand cleavage/activation site (denoted by "/") of rat PAR_2 (for B5, GPNSKGR/SLIGRLDTP). 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 PAR_2-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 PAR_2 at or beyond the tethered ligand activation site, KNRK-rPAR_2 cell suspensions were treated or not for 5 min at room temperature with enzyme concentrations that had been documented to activate rat PAR_2 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 95% ethanol (15 min), washed with PBS (twice for 5 s), and permeabilized with 0.2% Triton-X in PBS (5 min). Next, the cells were treated with a solution of 3% bovine serum albumin and 0.05% Tween 20 in PBS (5 min), washed with PBS (5 s), and incubated with either the SLAW-A (1:250 final dilution) or B5 (1:500 final dilution) antibodies in antibody diluting buffer (DAKO Diagnostics Canada Inc., Mississauga, Ontario, Canada) for 1 h in a humidity chamber. As a control, one set of cells was treated with a mixture of antisera preincubated with the immunizing peptide (SLAWLLGGPNSKGRGGYGGC for SLAW-A; GPNSKGRSLIGRLDTPYGGC for B5; each 1 μg/ml) instead of antibody alone. Finally, the cells were washed with PBS (three times for 5 s), incubated with anti-rabbit IgG Cy 3 conjugate (Sigma) (30 min), washed with PBS (5 s), air-dried, and mounted onto slides with 90% glycerol, 10% Tris (1 M, pH 8) mounting medium. 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 wtPAR$_2$ 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 PAR$_2$. 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 been validated previously 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 $CaCl_2$, 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 3×10^5 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$_1$ and PAR$_2$ in background HEK cells; PAR$_1$, PAR$_2$, and PAR$_4$ in HEKrPAR$_4$ 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 PAR$_1$ and PAR$_2$, the HEK cells were first desensitized by exposure to either the PAR$_1$AP, TFLLR-NH$_2$, or the PAR$_2$AP, SLIGRL-NH$_2$ (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 PAR$_1$ by TFLLR-NH$_2$ demonstrated the activation of PAR$_2$ by the proteinase, and the persistence of a calcium signal after desensitization caused by the PAR$_2$AP, SLIGRL-NH$_2$, indicated an activation of PAR$_1$. Furthermore, the complete disappearance of an hK-induced calcium signal after pre-desensitizing the HEK cells with TFLLR-NH$_2$ and SLIGRL-NH$_2$ in combination (100 μM each) or with 200 μM of the PAR nonselective agonist SFLLR-NH$_2$ 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 PAR$_1$ 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 PAR$_1$. In the continued presence of hK14, the cells were then challenged either with a PAR$_1$-activating concentration of thrombin (0.5 units/ml; 5 nM) or with a PAR$_1$-activating concentration of TFLLR-NH$_2$ (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" PAR$_1$ 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-NH$_2$, reflected a disarming of PAR$_1$. Alternatively, the diminished response to the peptide after hK14 treat-

TABLE 2

Scheme of proteolysis of PAR tethered ligand sequences by human kallikreins 5, 6, and 14

Major sites of cleavage detected by HPLC-mass spectrometry and by LC-MS analysis are denoted by bold upward arrows; minor sites of cleavage detected only by LC-MS/MALDI analysis of the complex peptide hydrolysis mixture are denoted by downward arrows. The tethered ligands for PAR_1, PAR_2, and PAR_4 are underlined.

	Trypsin	hK14	hK6	hK5
$hPAR_1$	N A T L D P **↑** R S F L L R N P N D K Y E	N A T L D P **↑** R S **↓** F L L **↑** R N P N D K Y E	N A T L D P **↑** R S **↓** F L **↓** L **↑** R N P N D K Y E	N A T L D P **↑** R S **↓** F L L R N P N D K Y E
$hPAR_2$	G T N R S S K G R **↑** S L I G K V D G T S H V T G K G V T	G T N R **↑** S S K G R **↑** S L I G K V D G T S H V T G K G V T	G T N R S S K G R **↑** S L I G K V D G T S H V T G K G V T	G T N R **↑** S S K G R **↑** S L I G K V D G T S H V T G K G V T
$hPAR_4$	G D D S T P S I L P A P R **↑** G Y P G Q V	G **↓** D D S T P S I L P A P R **↑** G Y P G Q V	G **↓** D D S T **↓** P S I L P A P R **↑** G Y P G Q V	G **↓** D **↓** D **↓** S T P S I L P A P R **↑** G Y P G Q V
$rPAR_2$	G P N S K G R **↑** S L I G R L D T P Y G G C	G P N S K G R **↑** S L I G R L D T P Y G G C	G P N S K G R **↑** S L I G R L D T P Y G G C	G P N S K G R **↑** S L I G R L D T P Y G G C
$rPAR_4$	L **↓** N E S K **↓** S P D K P N P R **↑** G F P G K P	L N E S K S P D K P N P R **↑** G F P G K P	L N E S K S P D K P N P R **↑** G F P G K P	L N E S K S P D K P N P R **↑** G F P G K P

ment represents *only* receptor desensitization and not disarming (36). In the same cell preparation, the PAR_1 calcium signal triggered by hK14 itself (monitored after desensitization of PAR_2 by prior treatment with SLIGRL-NH_2) was also measured. Because of the complexity of this procedure (which requires the use of nondesensitizing concentrations of hK14 to observe receptor disarming), it was not possible to do comparable experiments to measure with confidence the disarming by hK14 of either PAR_2 or PAR_4 for activation by either trypsin (PAR_2 and PAR_4) or thrombin (PAR_4).

Bioassay Procedures

Platelet Isolation and Aggregation Assay—Venous anticoagulated 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 puncture) by institutionally approved protocols. A platelet-rich plasma suspension was obtained (45) 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 platelets, the platelet suspension was supplemented with 0.8 μM prostaglandin I_2 (300 ng/ml), harvested by centrifugation (1,800 rpm; 620 × g_{max} for 10 min at room temperature), and resuspended in prostaglandin I_2 (0.8 μM)-supplemented calcium-free Tyrode's buffer, pH 7.4, of the following composition: NaCl (136 mM), KCl (3 mM), $NaHCO_3$ (12 mM), NaH_2PO_4 (0.4 mM), $MgCl_2$ (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 $CaCl_2$ and supplemented with indomethacin (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 fraction 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 subsequent 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 I_2 before use as 0.4-ml aliquots (2–3 × 10^8 platelets/ml) in calcium-replete (1 mM) Tyrode's buffer, pH 7.4. Light transmission was monitored with a dual channel aggregometer (Payton Scientific, Buffalo, NY). Agonists were added directly to the 0.4-ml suspension 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 concentration 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 PAR_2-activating peptides (46, 47). In brief, male Sprague-Dawley rats (250–300 g) or male C57/Bl6 mice (either wild-type or PAR_2 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 diameter) were cut for use in the bioassay. Aortic rings were equilibrated at 1 g resting tension for 1 h at 37 °C in a gassed (5% CO_2, 95% O_2) modified Krebs-Henseleit buffer, pH 7.4, of the follow-

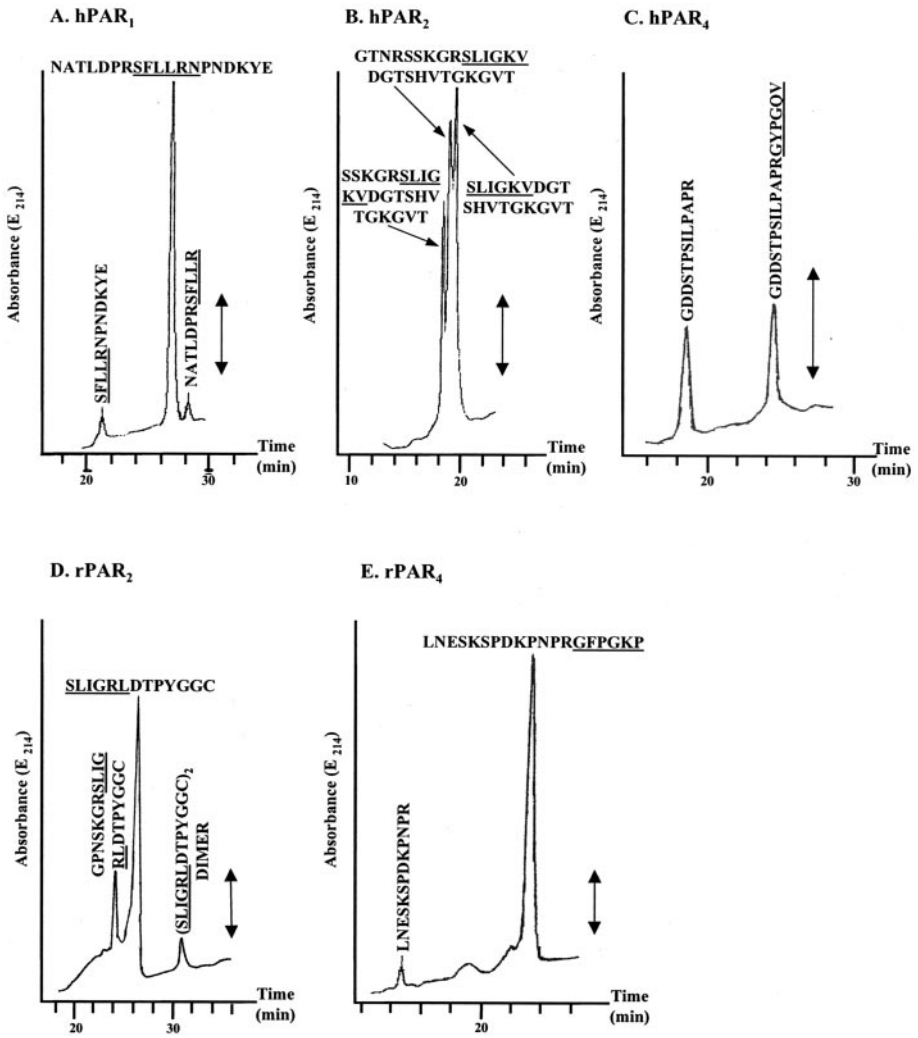

FIGURE 1. **HPLC separation and mass spectral analysis of PAR cleavage products generated by hK14.** Peptides representing the tethered ligand sequences of human PAR_1, PAR_2, and PAR_4 (*A–C*) and rat PAR_2 and PAR_4 (*D* and *E*) were subjected to proteolysis over a 30-min time period as described under "Materials and Methods." The peptides in the quenched reaction product were separated by HPLC analysis, and peptides in the peak fractions (absorbance, E_{214}) were collected and subjected to mass spectral analysis. The sequences of the peptides deduced from the mass spectral data are indicated next to each peak. *Underlined* amino acid residues represent the key receptor activating tethered ligand sequences. The *double arrows* in each panel represent 0.01 absorbance units.

ing composition: NaCl (118 mM), KCl (4.7 mM), $CaCl_2$ (2.5 mM), $MgCl_2$ (1.2 mM), $NaHCO_3$ (25 mM), KH_2PO_4 (1.2 mM), and glucose (10 mM). The relaxant actions of trypsin, the hKs, or the PAR-activating peptides (about 2 μM SLIGRL-NH_2 or 5 μM TFLLR-NH_2) were measured in endothelial intact aortic rings that were pre-contracted with 1 μM phenylephrine. A relaxant response to 10 μM acetylcholine (a 60–95% reduction of the tension generated by 1 μM 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 μM 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_1 cleavage sequence, NATLDPRSFLLR (Fig. 1, *tracing A*), indicated that hK14 cleaved within the tethered ligand sequence of $hPAR_1$ 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 PAR_2 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 PAR_2 peptide was more rapid, with the amount of receptor-activating peptide released at 30 min (SLIGRL . . .) 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 PAR_2. 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 PAR_4 peptides. Peptide hydrolysis by these two hKs released significant amounts of the receptor-activating peptide for human and rat PAR_2 and small amounts of the $hPAR_1$-activating peptide (not shown). A peptide suggesting a disarming cleavage of PAR_1 (Fig. 1, *tracing A, 3rd peak* on the *right*) was not observed for the cleavage of the $hPAR_1$ peptide by either hK5 or hK6. The human PAR_4 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 PAR_4-activating peptide was detected by HPLC analysis from the rat-derived PAR_4 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 PAR_1, PAR_2, and PAR_4 synthetic peptide sequences to generate receptor-activating peptides, with a predominant cleavage of the $hPAR_2$ peptide compared with PAR_1 and PAR_4. Although hK5 and hK6 cleavage of the rat PAR_2-derived sequence also yielded significant amounts of a receptor-activating peptide, the $rPAR_4$ 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 PAR_1 was not studied. This analysis (Table 2) confirmed the release by hK5, -6, and -14 of the tethered ligand sequences from human PAR_1, PAR_2, and PAR_4 and from rat PAR_2. A minor cleavage of rat PAR_4 to yield its receptor-activating sequence was also suggested (Table 2). In addition, the analysis confirmed the cleavage of the $hPAR_1$ 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 PAR_2 or at a lysine C-terminal to the tethered ligand of rat PAR_4, to yield the peptide sequence LNESKSPDKPNPRGFPGK). 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 peptides 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 PAR_1 or the GY/PG site in $hPAR_4$ (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 PAR_1, PAR_2, and PAR_4 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 proteinase contaminants in the hK preparations. The potential physiological impact of the minor sites of cleavage is difficult to assess.

Evaluation of hK-mediated PAR_2 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 PAR_2 at the cell surface, we used an immunohistochemical approach to monitor PAR_2 cleavage in intact rat PAR_2-expressing KNRK cells ($KNRKrPAR_2$), 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 in intact tissues (35, 40). The analysis depends upon the reactivity of two targeted antisera (B5 and SLAW-A) with two distinct domains of rat PAR_2 (35). The B5 antiserum detects the cleavage-activation sequence of PAR_2, whereas the SLAW-A antiserum detects only the pre-cleavage sequence. When $KNRKrPAR_2$ cells were incubated with hK14 under conditions that would

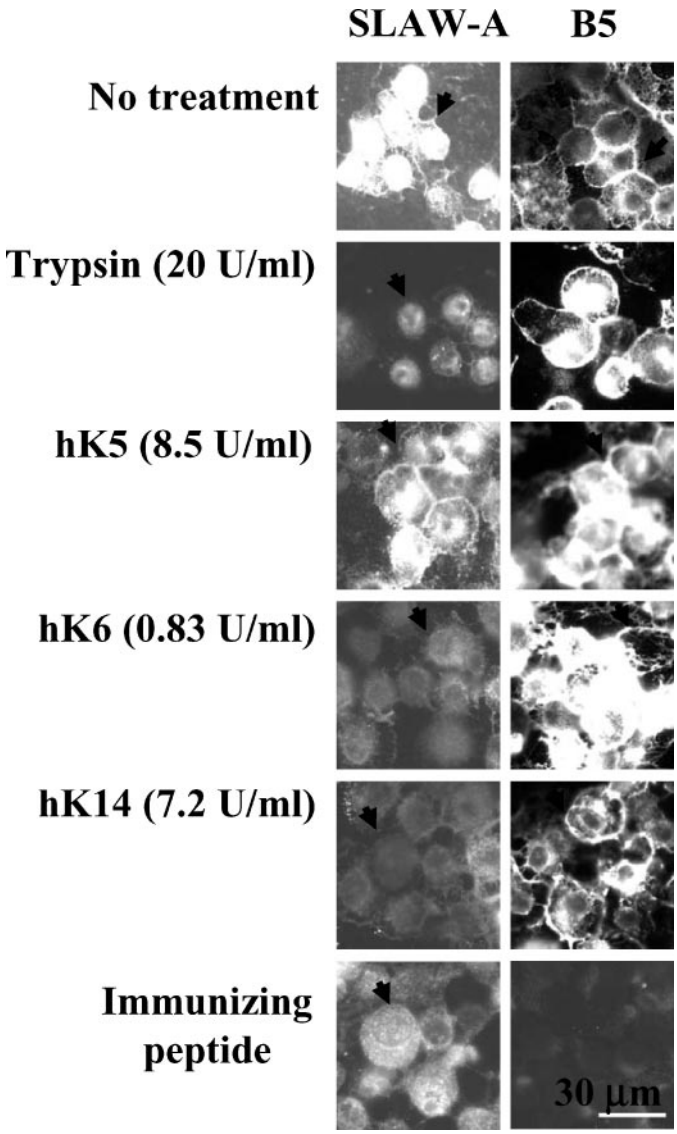

FIGURE 2. **Kallikrein-mediated removal of the pre-cleavage sequence of rat PAR_2 in intact cells.** KNRK cells expressing rat PAR were treated or not with either trypsin or hK5, -6, and -14 under conditions that would have produced a calcium signal. The presence or absence of the pre-cleavage epitope detected by the SLAW-A antibody (*left-hand panels*) and the tethered ligand portion of PAR_2 detected by the B5 antibody (*right-hand panels*) were then monitored, as outlined under "Materials and Methods." Pre-adsorption of the SLAW-A and B5 antisera with the immunizing peptides eliminated the PAR_2-specific immunoreactivity in enzyme-untreated cells (*bottom two panels*). The micrograph is representative of five or more randomly selected optical fields used for the morphometric analysis calculations that were based on three independently conducted experiments, as outlined under "Materials and Methods." *Scale bar*, 30 μm.

result in calcium signaling (see below), morphometric analysis of the immunoreactive cells revealed that there was a loss of reactivity with the SLAW-A antiserum (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 PAR_2 had occurred, removing the epitope(s) reacting with the SLAW-A antiserum, but cleavage at other downstream sites (either tryptic or chymotryptic), which would have released the epitope(s) reacting with the B5 antiserum, had evidently not occurred. Treatment of the $KNRKrPAR_2$ 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 antiserum 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 antiserum 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 PAR_1 and PAR_2

To determine whether hK14 cleaves and activates PAR_2, we measured its effects on calcium signaling by both the human and rat receptors expressed in intact cells. We first tested $KNRKrPAR_2$ cells that were used for the immunohistochemical monitoring of PAR_2 activation. These cells express only rat PAR_2 and not the other PARs. In the $KNRKrPAR_2$ cells, hK5, -6, and -14 were all able to generate a calcium signal comparable with that of trypsin (Fig. 3*A*, *tracing A*) but did not produce a signal in the nontransfected KNRK cells at the same enzyme concentrations (not shown). Furthermore, pre-desensitizing the PAR_2-generated calcium signal in the $KNRKrPAR_2$ cells by pre-exposure to a high concentration of the PAR_2 agonist, $SLIGRL\text{-}NH_2$ (50 μM), prevented the subsequent kallikrein-triggered calcium signal (Fig. 3*A*, *tracing B*). Finally, in the KNRK cell line expressing the rat PAR_2(R36A) mutant receptor, in which the R36A mutation abrogates trypsin-mediated (but not $SLIGRL\text{-}NH_2$-triggered) activation of PAR_2 (Fig. 3*A*, *tracing C*, first result on the *left*) (35), none of kallikreins 5, 6, or 14 caused a calcium signal (Fig. 3*A*, *tracing C, right-hand* results) at concentrations that did so in the wild-type $KNRKrPAR_2$ cells (Fig. 3*A, tracing A, right-hand* results). As expected, the selective PAR_2-activating peptide, $SLIGRL\text{-}NH_2$, did elicit a calcium signal in the PAR_2(R36A) cells (Fig. 3*A*, *tracing C, right-hand* tracings). Thus, kallikreins 5, 6, and 14 activate rat PAR_2 at the same Arg^{36}/Ser^{37} site as trypsin to expose the tethered ligand sequence. The concentration-effect curve for hK14-mediated activation of PAR_2 calcium signaling was compared with the curves for hK6 and trypsin in the $KNRKrPAR_2$ cells (Fig. 3*B*). The relative potencies (inverse of the EC_{50} 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 EC_{50} than that of hK14, indicating a higher potency for PAR_2 activation, the maximum calcium signal generated by hK14 was much greater than that for hK6 (Fig. 3*B*), 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 $KNRKrPAR_2$ cell line that expresses a high abundance of only one of the PARs, the human HEK cells

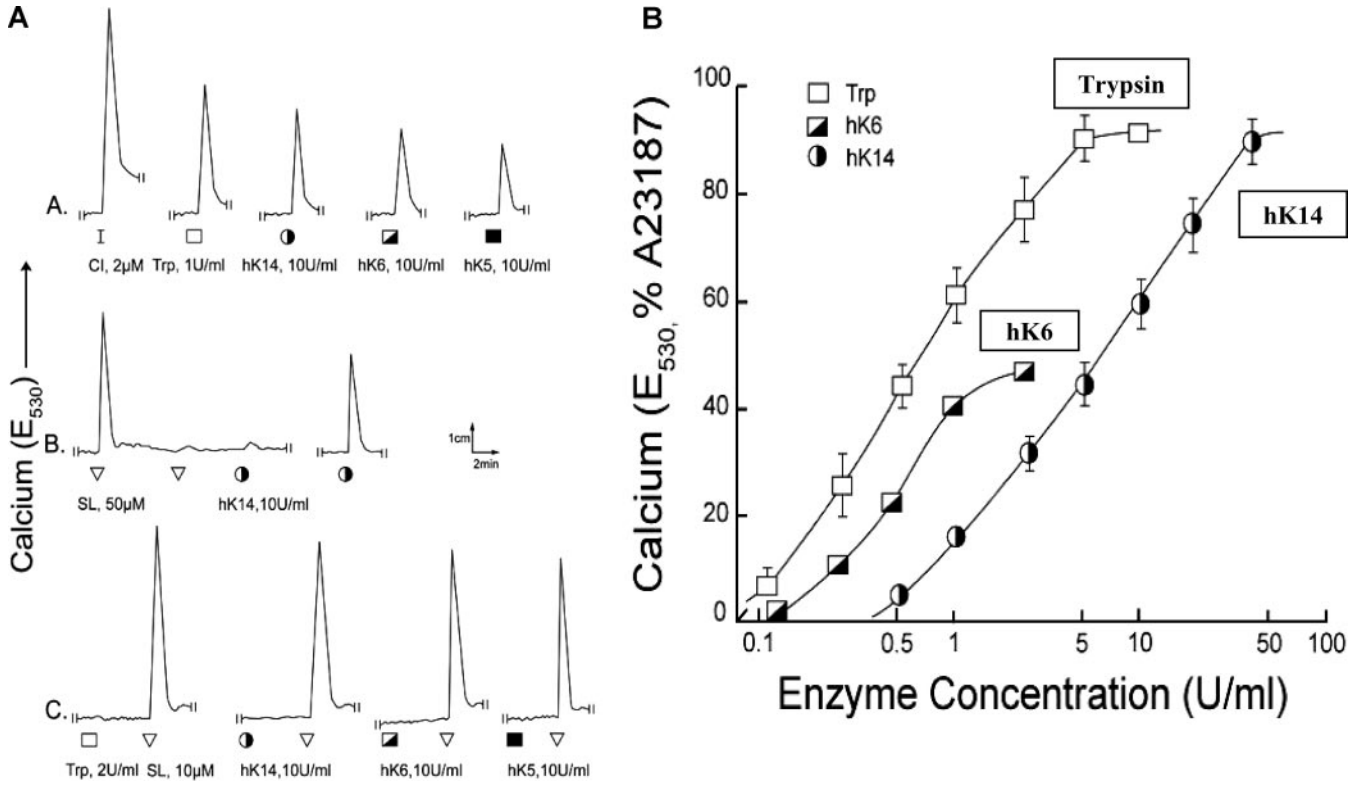

FIGURE 3. *A,* mobilization of intracellular calcium by hKs, trypsin, and SLIGRL-NH_2 in KNRKrPAR$_2$ and KNRKrPAR$_2$(R36A) cells. *Tracing A,* representative calcium response data triggered by trypsin (Trp, 1 unit/ml; □), hK14 (◑), hK6 (◪), hK5 (■), and calcium ionophore (*Cl*, I). *Tracing B,* desensitization 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.

constitutively express both PAR$_1$ and PAR$_2$ (but not PAR$_4$) 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. 4*A*, *tracing A*). The ability of hK14 to activate either of PAR$_1$ and PAR$_2$ individually was assessed using a receptor desensitization assay (Fig. 4*A*, *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. 4*A*, *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. 4*A*, *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. 4*A*, *tracing D*). From the peak heights of the calcium signals observed after pre-desensitization of either PAR$_1$ (PAR$_2$ signal seen in Fig. 4*A*, *tracing B*) or PAR$_2$ (a much smaller PAR$_1$ signal seen in Fig. 4*A*, *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. 4*A*, 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. 4*B*); 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. 4*B*). As shown in Fig. 4*B*, the potency of hK14 for activating a calcium signal via PAR$_2$ (*middle curve* in Fig. 4*B*) was close to its potency for signaling via PAR$_1$ (*lower curve*, Fig. 4*B*), 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. 4*A*, *tracings A*

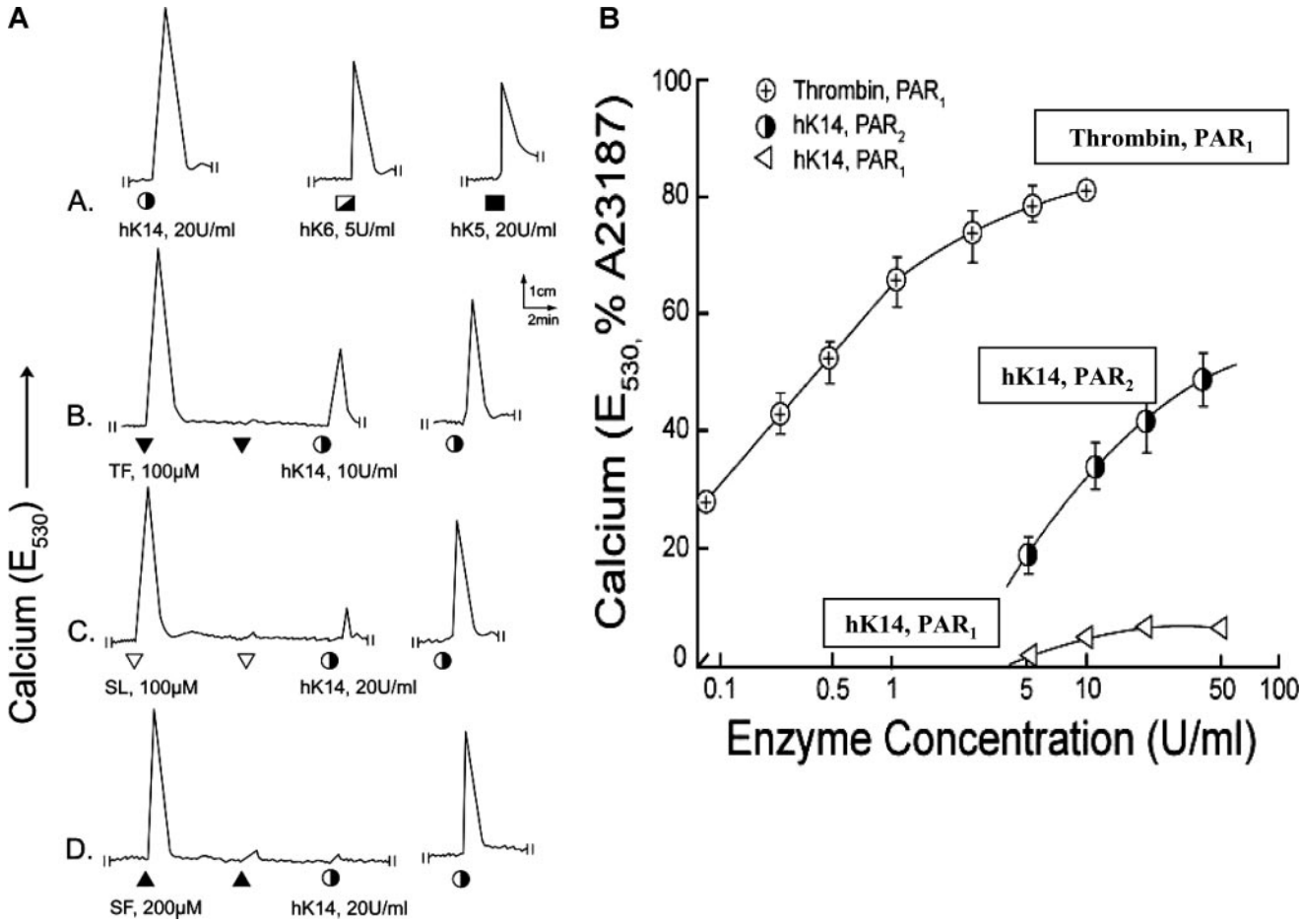

FIGURE 4. *A,* mobilization of intracellular calcium by hK5, -6, and -14 in HEK cells; reduction in signaling by prior desensitization of PAR_1 and PAR_2. An upward deflection (*arrow* on *ordinate*) indicates an increase in intracellular calcium. *Tracing A,* tracings show representative combined PAR_1/PAR_2-mediated increases in HEK cell intracellular calcium caused by hK14, -6, and -5. *Tracing B,* PAR_2-mediated signaling by hK14 in HEK cells pretreated with TFLLR-NH_2 to desensitize HEK PAR_1. *Tracing C,* PAR_1-mediated signaling by hK14 in HEK cells pretreated with SLIGRL-NH_2 to desensitize HEK PAR_2. *Tracing D,* lack of hK14 signaling in HEK cells pretreated with SFLLR-NH_2 to co-desensitize both PAR_1 and PAR_2. 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. B,* concentration-effect curves for hK14-mediated mobilization of intracellular calcium in HEK cells by selectively activating either PAR_1 (◁) or PAR_2 (◑); comparison with thrombin-mediated activation of PAR_1 (⊕). To monitor hK14 signaling selectively either via PAR_1 or via PAR_2, HEK cells were first desensitized by either the selective PAR_2 agonist, SLIGRL-NH_2 (revealing signaling by PAR_1: ◁, *bottom curve*) or by the selective PAR_1 agonist, TFLLR-NH_2 (revealing activation by PAR_2 (◑, *middle curve*). Calcium signaling by increasing concentrations of hK14 in the pre-desensitized cells was monitored. The signaling of hK14 via PAR_1 (*bottom curve*) can be compared with signaling by thrombin via PAR_1 (⊕, *top curve*). In the HEK cells, thrombin can signal only via PAR_1, because it is unable to activate PAR_2. 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.

and *C*). In contrast, the maximum calcium signal generated by the selective activation of PAR_1 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 PAR_1 with thrombin (*top curve* in Fig. 4*B*). The considerably lower maximum PAR_1-derived calcium signal caused by hK14 relative to that caused by thrombin (or, relative to the TFLLR-NH_2 peptide-stimulated calcium signal, compare the *left-hand portion* of *tracing B* in Fig. 4*A* with the hK14-generated signal in *tracing C* of Fig. 4*A*) suggested a combined activation/disarming of PAR_1 by hK14 (see below).

Thus, hK5, -6, and -14 activate calcium signaling in the HEK cells, with hK14 causing a strong signal via PAR_2 at concentrations about 8-fold higher than those at which trypsin activates PAR_2. Also, like trypsin, hK14 signals predominantly via PAR_2 in the HEK cells, causing only a small signal (relative to that triggered by thrombin or TFLLR-NH_2) via PAR_1 activation.

hK-mediated Disarming/Inhibition of HEK Cell PAR_1

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 PAR_1 activation, relative to the maximum PAR_1 signal caused by either thrombin or the PAR_1-activating peptide, TFLLR-NH_2, pointed to a disarming/inhibition of PAR_1 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 PAR_1. As shown in Fig. 5*A*, pretreatment of HEK cells with hK14 at a concentration that did not by itself desensitize the calcium signal caused by TFLLR-NH_2 (*i.e.* no desensitization of PAR_1; Fig. 5*A*, *tracing B,* and *lower panel,*

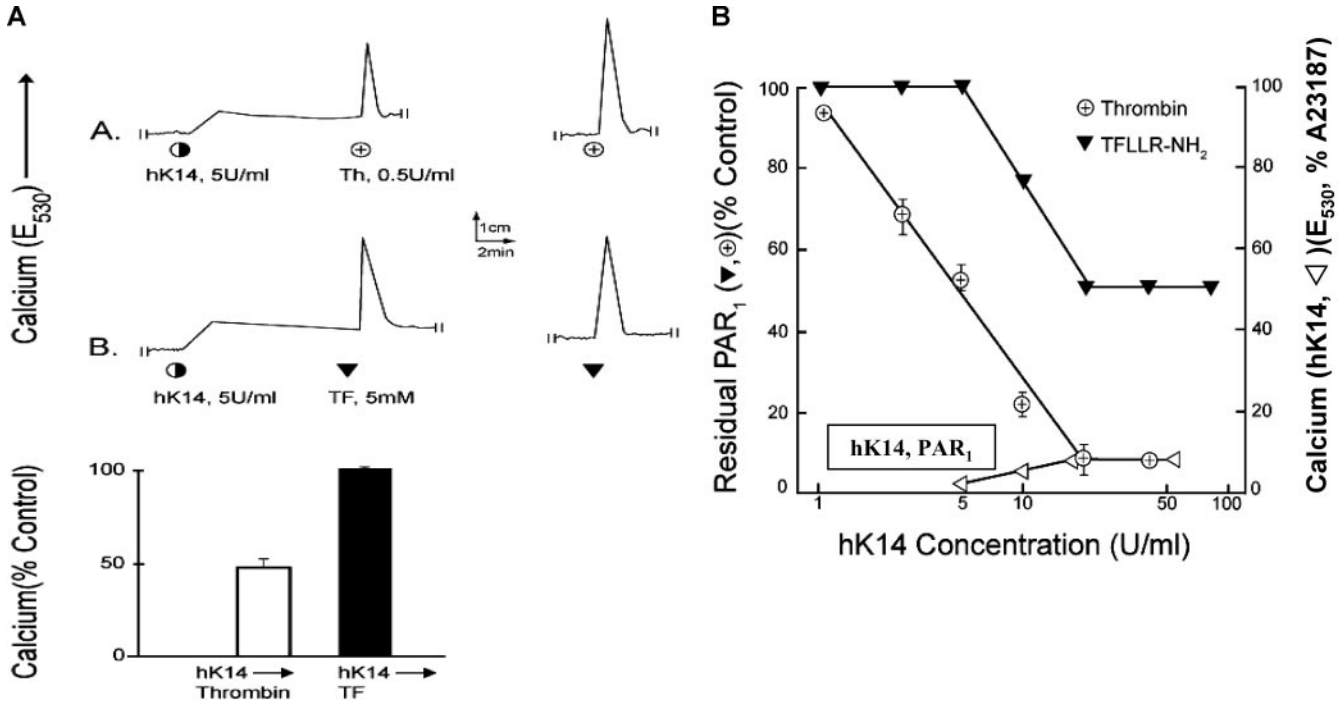

FIGURE 5. *A,* disarming by hK14 of thrombin (*tracing A*) but not TFLLR-NH_2 (*tracing B*) activation of PAR_1 in HEK cells. *Upper,* cell suspensions in which calcium signaling was monitored were first exposed to a submaximally effective concentration of hK14, followed by the addition of a test concentration of either thrombin (*tracing A*) or the selective PAR_1-activating peptide, TFLLR-NH_2 (*tracing B*). The responses of thrombin and TFLLR-NH_2 in the hK14-pretreated cells were compared with the control responses in untreated cells (*right-hand portions* of *tracings A* and *B*). *Lower,* the histograms, representing the averaged data from experiments like those depicted in *tracings A* and *B* (±S.E., *bars, n* = 3), show the reduction in thrombin (*open histogram*) but not TFLLR-NH_2 (*solid histogram*)-mediated calcium signaling in cells that were pretreated with hK14. The calcium signaling responses after hK14 treatment were expressed as a percentage (% control) of the signal observed in the same cell suspension that had not been pretreated with hK14. Data represent the average of three experiments with independently grown HEK cell cultures. *B,* disarming by hK14 of thrombin activation of PAR_1 (⊕) and hK14-mediated desensitization of TFLLR-NH_2 PAR_1 signaling in HEK cells; concentration-effect curves for hK14. HEK cell suspensions prepared for calcium signaling were first exposed at 24 °C to increasing concentrations of hK14, and the calcium signal was allowed to return to baseline, with re-filling of the intracellular calcium stores (10 min). Test concentrations (EC_{50}) of either thrombin (0.5 units/ml; ⊕, *middle curve*) or TFLLR-NH_2 (5 μM; ▼, *top curve*) were then added, and the residual PAR_1-mediated calcium signal (▼, ⊕) was monitored as a percentage (% control) of the calcium signal generated in identical cell suspensions that had not been pretreated with hK14. For comparison, the concentration-effect curve for PAR_1-mediated signaling by hK14 alone (◁, from Fig. 4*B*) in cells that were first PAR_2 pre-desensitized with SLIGRL-NH_2 is shown in the *bottom curve.*

right-hand bar), reduced the subsequent signal caused by thrombin activation of PAR_1 (Fig. 5*A*, *tracing A,* and *lower panel, left-hand bar*). It is important to note in interpreting this experiment using the HEK cells expressing both PAR_1 and PAR_2 that unlike trypsin or hK14, thrombin is *not* able to activate a calcium signal in the HEK cells via PAR_2. The concentration-effect curve for the ability of hK14 to disarm the ability of thrombin to activate HEK cell PAR_1 is shown in Fig. 5*B*. 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-NH_2. 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-NH_2 was retained (not shown). This result indicated a disarming of PAR_1, 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 EC_{50} value for the ability of hK14 to disarm/inhibit PAR_1 (about 4.5 units/ml, *left-hand upper curve* in Fig. 5*B*) was lower than its EC_{50} value (about 10 units/ml, *right-hand, upper curve* in Fig. 5*B*) for causing a desensitization of the selective activation of PAR_1 in the same cells by TFLLR-NH_2. Thus, in cells co-expressing PAR_1 and PAR_2, hK14, and presumably the other hKs, can have complex actions, activating PAR_2 and, depending on the concentration, either disarming/inhibiting or activating PAR_1.

hK-mediated Activation of PAR_4

Rat Platelet Aggregation Assay—Because rat platelets possess PAR_4 but neither PAR_1 nor PAR_2, thrombin-mediated aggregation can be attributed to PAR_4 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 PAR_4. Like thrombin and trypsin that act *via* PAR_4 in this preparation, hK14 caused aggregation. Surprisingly, however, neither hK5 nor hK6 were able to do so at concentrations that could activate PAR_2 (20 units/ml: Fig. 6). The concentration-effect curve for hK14-mediated rat platelet aggregation ($EC_{50} \cong$ 14 units/ml) showed that its potency was about 2-fold lower than that of trypsin ($EC_{50} \cong 7$ units/ml) for activating rat platelet PAR_4 (Fig. 6, *left-hand curve*).

hK-mediated Activation of Rat PAR_4 Expressed in HEK Cells—To assess further the ability of hK14 to activate rat PAR_4, we used HEK cells expressing recombinant rat PAR_4 in addition to the constitutive expression of human PAR_1 and PAR_2. Activa-

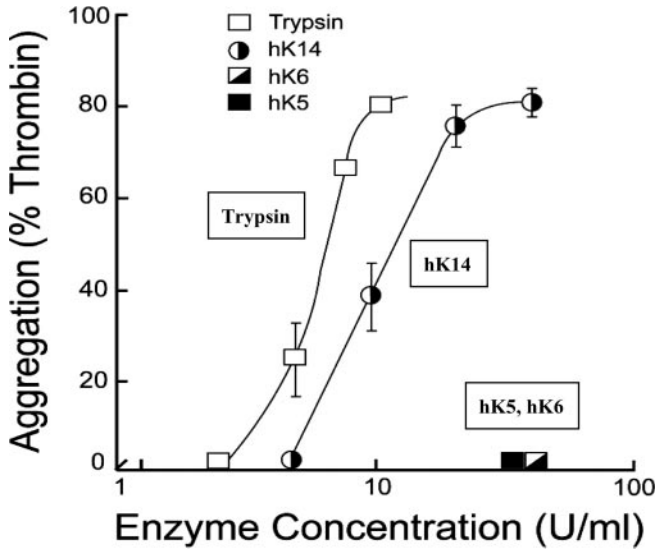

FIGURE 6. **Aggregation of rat platelets by thrombin, trypsin, and hK14 but not by hK5 and -6; concentration-effect curves for hK14.** Rat platelet suspensions were exposed to the indicated concentrations of enzymes, and aggregation was monitored as an increase in light transmission (% maximum change relative to maximal light transmitted in the absence of platelets) as described under "Materials and Methods." Aggregation of rat platelet suspensions exposed to increasing concentrations of either trypsin (□) or hK14 (◑) was monitored and expressed as a percentage (% thrombin) of the maximal aggregation caused in the same platelet suspensions by 1 unit/ml thrombin. Neither hK6 nor hK5 caused aggregation at concentrations equivalent to hK14. Data at each enzyme concentration represent the averages of eight or more aggregation measurements (±S.E., *bars*) done with separately prepared platelet suspensions.

tion of PAR_4 with the PAR_4-selective activating peptide, AYPGKF-NH_2 (which cannot activate either PAR_1 or PAR_2), caused an increase in intracellular calcium (Fig. 7*A*, *tracing B*) in cells that had already been desensitized with SFLLR-NH_2 to eliminate any signaling by either PAR_1 or PAR_2. In cells that were first desensitized with SFLLR-NH_2, which simultaneously silences signaling by PAR_1 and PAR_2, hK14 yielded a robust calcium signal (Fig. 7*A*, *tracing A*). In the same type of assay, cells pre-desensitized by the combined agonists of PAR_1, PAR_2, and PAR_4 (SFLLR-NH_2 plus AYPGKF-NH_2) no longer yielded a calcium signal in response to hK14 (Fig. 7*A, tracing B, right-hand portion*). Furthermore, HEK cells that were not transfected with the rat PAR_4 construct, upon being desensitized with SFLLR-NH_2 (to silence both PAR_1 and PAR_2), no longer responded to hK14 (data not shown and result shown in Fig. 4*A*, *tracing D*). Thus, unequivocally, hK14 was able to activate PAR_4 in the HEK-rPAR_4 cells and did not cause its calcium signal via a non-PAR mechanism. To evaluate the sensitivity of rat PAR_4 to activation by hK14, HEK-rPAR_4 cells were PAR_1/PAR_2 predesensitized by treatment with a relatively high concentration of SFLLR-NH_2 and were then exposed to increasing concentrations of hK14 (Fig. 7*B*, *right-hand curve*). The potency of hK14 in this assay (EC_{50} ≅ 15 units/ml) (Fig. 7*B*, *right-hand curve*) was in the same range as that of trypsin (EC_{50} ≅ 9 units/ml) (Fig. 7*B*, *left-hand curve*). These results for hK14-mediated activation of rat PAR_4 expressed in HEK cells were entirely in keeping with the ability of hK14 to activate PAR_4-dependent rat platelet aggregation (above) and to release a PAR_4-acti-

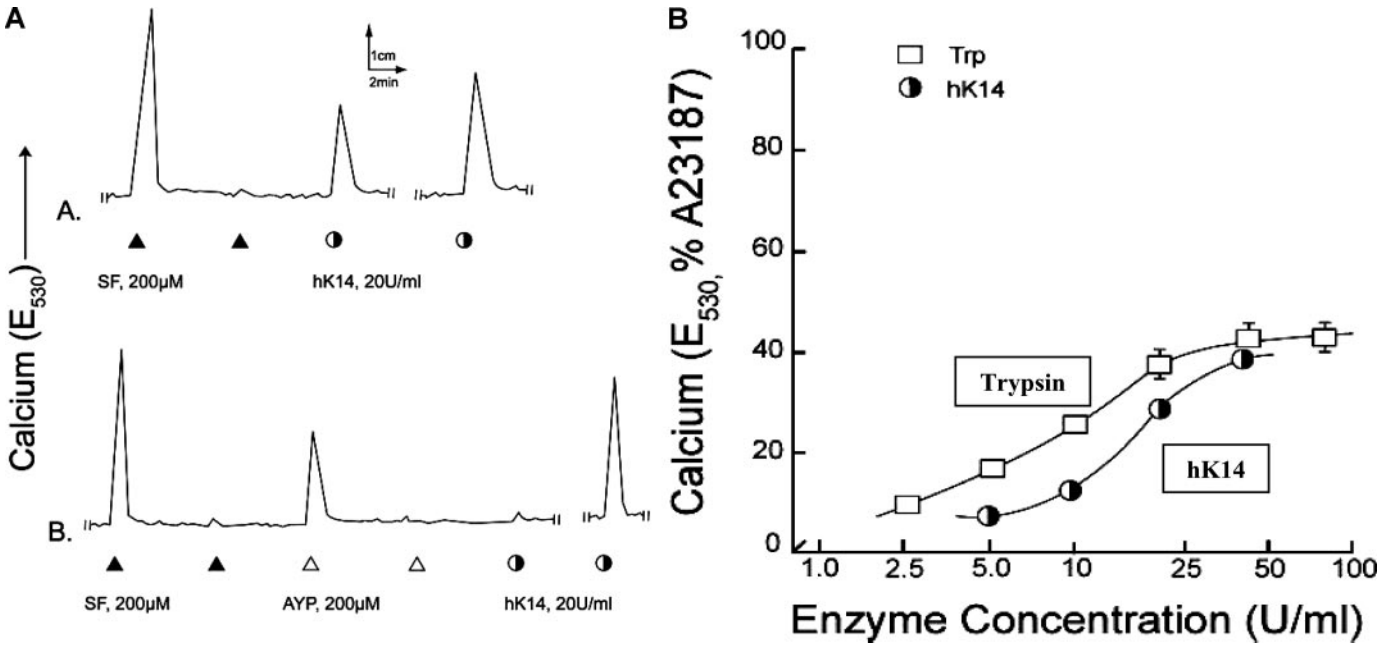

FIGURE 7. *A,* hK14 activation of rat PAR_4 calcium signaling in HEK-rPAR_4 cells. Representative tracings show the following. *Tracing A,* hK14-triggered mobilization of intracellular calcium via PAR_4 in HEK-rPAR_4 cells PAR_1/PAR_2 desensitized by pretreatment with supra-maximal concentrations of the nonselective PAR_1/PAR_2 agonist SFLLR-NH_2. The hK14-mediated calcium signal in nondesensitized cells is shown on the right. *Tracing B,* elimination of hK14 signaling in HEK-rPAR_4 cells PAR_1/PAR_2/PAR_4 desensitized by pretreatment with supra-maximal concentrations of the PAR_1/PAR_2 agonist, SFLLR-NH_2, plus the selective PAR_4 agonist AYPGKF-NH_2. The tracings are representative of three or more independently conducted experiments using separately grown cell cultures. *B,* activation of rat PAR_4 in HEK-rPAR_4 cells by hK14 and trypsin concentration-effect curves for mobilization of intracellular calcium. HEK-rPAR_4 cells prepared for calcium signaling experiments were first PAR_1/PAR_2 desensitized with a supra-maximal concentration of SFLLR-NH_2, and the calcium signals generated by increasing concentrations of either trypsin (Trp, □) or hK14 (◑) were then monitored and expressed as a percentage (% A23187) of the calcium signal caused by 2 μM of the ionophore A23187 in the same cell suspension. Data points at the indicated enzyme concentrations represent the average values (±S.E., *bars*) obtained from four or more replicate cell suspensions derived from independently grown cell cultures. *Error bars* smaller in magnitude than the symbols are not shown.

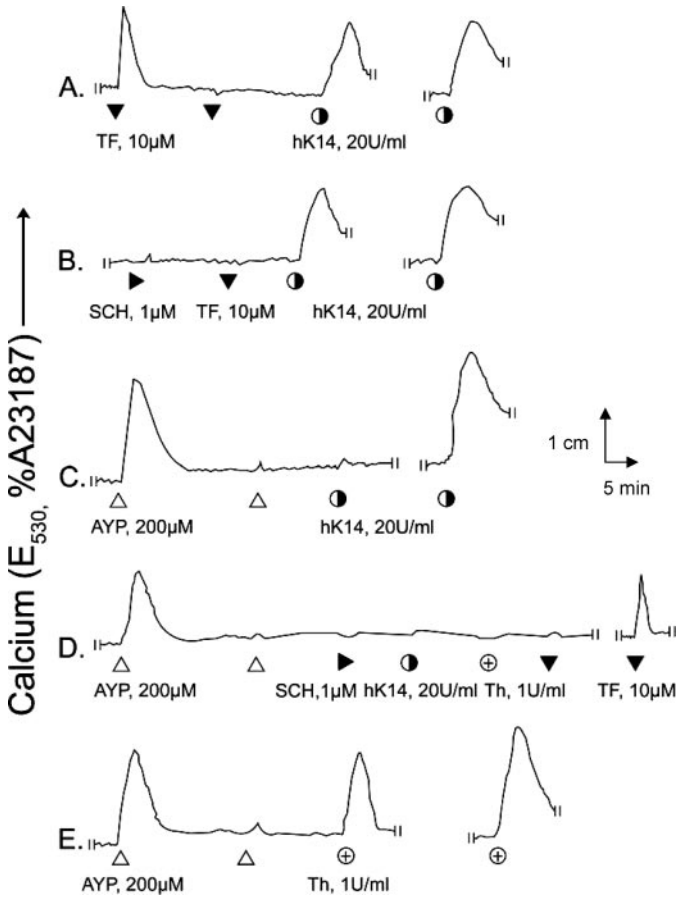

FIGURE 8. **Mobilization of intracellular calcium in human platelets by hK14 signaling via PAR_4 (*tracings A* and *B*) and PAR_1 (*tracing C*); comparison with thrombin activation of PAR_1.** *A* and *B*, selective signaling of hK14 via PAR_4. Platelet PAR_1 signaling was eliminated either by prior desensitization of PAR_1 with TFLLR-NH_2 (*TF*, ▼, *tracing A*) or by blockade of PAR_1 with SCH 79797 (SCH, ▶, *tracing B*). Residual signaling of hK14 via PAR_4 was then monitored. *C*, selective signaling by hK14 via PAR_1. Platelet PAR_4 signaling was eliminated by prior desensitization with the PAR_4-selective agonist AYPGKF-NH_2 (*AYP*, △), revealing a small residual PAR_1 signal triggered by hK14. *D*, lack of signaling by thrombin and hk14 after concurrent desensitization of PAR_4 and PAR_1 with AYPGKF-NH_2 and SCH 79797 (PAR_1 antagonist). *E*, selective signaling of thrombin via PAR_1. Platelet PAR_4 signaling was eliminated by prior desensitization with the PAR_4-selective agonist, AYPGKF-NH_2 (△), revealing substantial residual PAR_1 signal triggered by thrombin. The *tracings* are representative of three or more identical experiments done with independently harvested human platelet preparations.

vating sequence from the synthetic rat PAR_4-derived tethered ligand peptide (Table 2).

hK-mediated Activation of PAR_4 in Human Platelets—To assess the ability of hK14 to activate human PAR_4, 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 PAR_1 and PAR_4 in the same platelet aliquot. In the human platelet, which does not possess PAR_2, aggregation is triggered by the combined action of PAR_1 and PAR_4 (4). That said, activation of PAR_1 and PAR_4 can have distinct effects on platelet function (51), and we therefore wished to determine whether hK14 might differentially activate PAR_1 and PAR_4 in the human platelet preparation. As shown in Fig. 8, the receptor-selective activating peptides for PAR_1 (TFLLR-NH_2, Fig. 8, *tracing A*) and PAR_4 (AYPGKF-NH_2, 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 PAR_1 antagonist SCH 79797, to eliminate either TFLLR-NH_2 or thrombin signaling via PAR_1 (Fig. 8, *left portion* of *tracing B,* and data not shown for thrombin), or with a PAR_1-desensitizing concentration of TFLLR-NH_2 (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 PAR_1-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 PAR_4. In keeping with this result, treatment of the platelets with a PAR_4-desensitizing concentration of AYPGKF-NH_2 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-NH_2 (on average ($n = 4$) 10% of the signal generated in nondesensitized platelets) was presumably because of PAR_1 activation. Finally, the combined treatment of the platelets with the PAR_1 antagonist SCH 79797 in combination with a desensitizing concentration of AYPGKF-NH_2 eliminated the hK14-triggered calcium signal as well as the signal generated by either thrombin or TFLLR-NH_2 (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 PAR_4 desensitization to reduce the hK14 signal by about 90% (Fig. 8, *tracing C*), the human platelet signal generated by thrombin after PAR_4 desensitization, because of PAR_1 activation, was reduced by only about 20% (Fig. 8, *tracing E*). Thus, in contrast with the thrombin-triggered signal generated largely by PAR_1 activation, calcium signaling in human platelets driven by hK14 appeared to result primarily (about 90%) from activation of PAR_4 and not PAR_1 (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 PAR_1 or PAR_4. 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 hKs in terms of activating human (and rat) PAR_4.

In sum, the calcium signaling experiments showed that in human platelets, as in the rPAR_4-expressing HEK cells (Fig. 7*A*), hK14 can preferentially activate PAR_4 rather than PAR_1, which is in contrast disarmed/inhibited by hK14. This selectivity of hK14 for PAR_4 over PAR_1 in the platelet is the opposite of the selectivity observed for thrombin, which preferentially activates human platelet PAR_1, although also able to activate PAR_4 at higher concentrations (4).

Vascular Bioassays

Aorta Ring Relaxation Assays Using Rat and Murine Vascular Tissue—Activation of either PAR_1 or PAR_2 (46, 52) but not PAR_4 (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

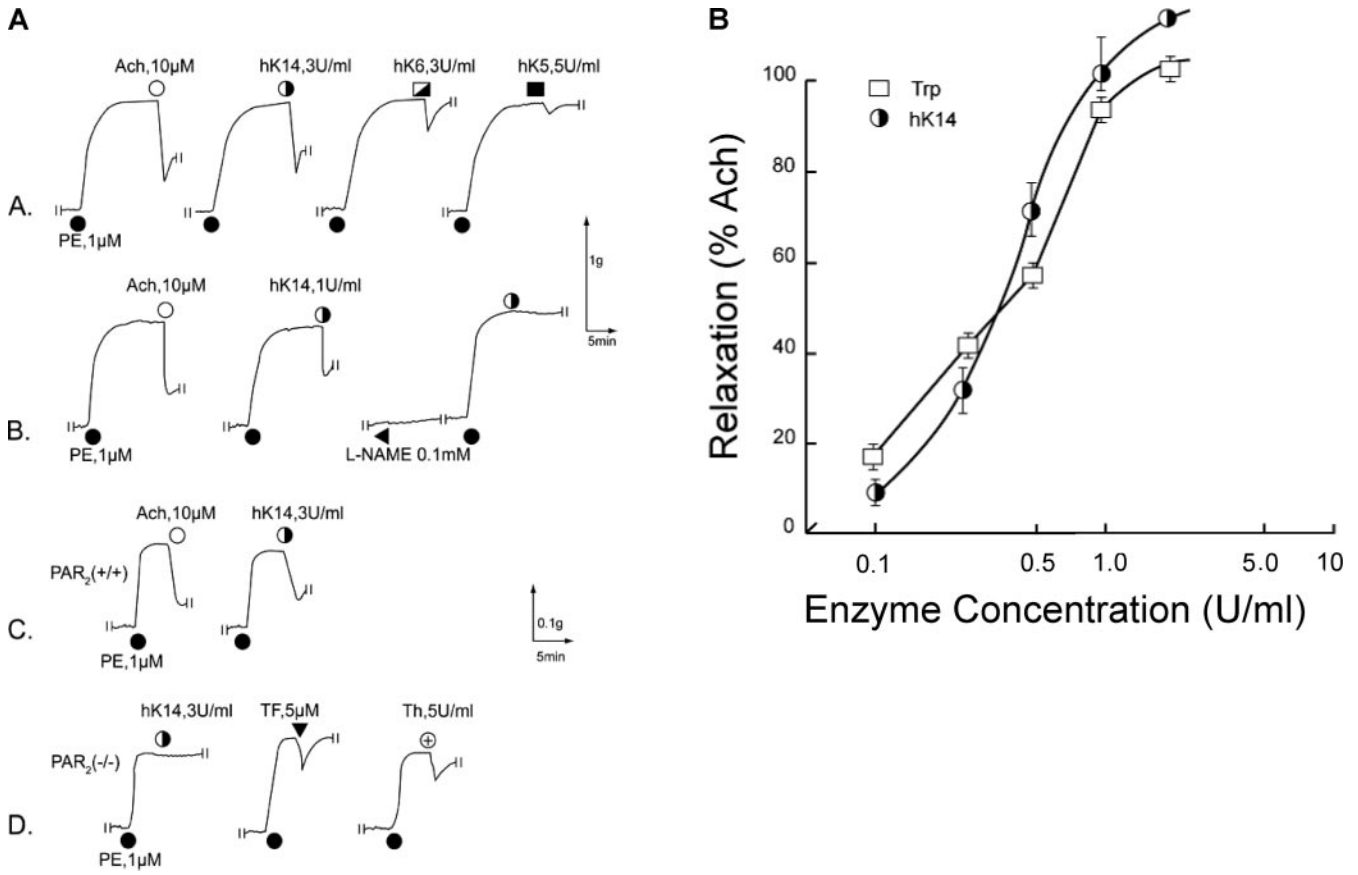

FIGURE 9. *A,* hK-mediated relaxation of rat and murine aorta preparations; lack of effect in tissue from PAR_2 null mice. *Tracings A* and *B,* rat aorta relaxation. *Tracing A,* representative tracings for the relaxant actions of hKs14, -6 and -5 in rat aorta tissue with an intact endothelium (relaxant response to 10 μM acetylcholine, ○). *Tracing B,* relaxant effect of hK14 is blocked by the NO-synthase inhibitor, L-NAME (◀). *Tracings C* and *D*, murine aorta relaxation. *Tracing C,* representative tracing for relaxation caused by hK14 in an endothelium-intact murine aorta preparation (relaxant response to 10 μM acetylcholine, ○). *Tracing D,* lack of relaxation caused by hK14, with relaxation caused by thrombin and the PAR_1-activating peptide TFLLRN-NH_2 (*TF*) in aorta tissue from PAR_2-null mice. *B,* concentration-effect curves for hK14-mediated relaxation in wild-type C57Bl murine aorta, compared with trypsin concentration-effect curve. The relaxant responses triggered by increasing concentrations of either trypsin (□) or hK14 (◑) in phenylephrine-constricted endothelium-intact C57Bl murine aorta preparations were monitored and expressed as a percentage (% acetylcholine (*Ach*)) of the relaxant action of 10 μM acetylcholine in the same tissue preparation. Values at each enzyme concentration represent the averages (±S.E., *bars*) of four or more independent measurements on tissue preparations obtained from three or more animals.

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. 9*A*, *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. 9*A*, *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-contracted aorta from wild-type mice (Fig. 9*A*, *tracing C*) and PAR_2 null mice (Fig. 9*A*, *tracing D*). The concentration-effect curve for this relaxant action of hK14 in the wild-type murine aorta preparation was also determined (Fig. 9*B*), 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. 9*A*, *tracing C*). The potency of hK14 for causing a relaxant response in the murine aorta preparation was close to that of trypsin ($EC_{50} \cong 0.5$ units/ml; Fig. 9*B*). Concentrations of hK14 toward the plateau of the concentration-effect curve (3 units/ml) failed to cause relaxation in pre-constricted tissues from the PAR_2 null mice, which otherwise relaxed in response to PAR_1 activation by TFLLR-NH_2 or thrombin (Fig. 9*A*, *tracing D*). Thus, hK14 relaxed the mouse aorta by the selective activation of PAR_2 and not via PAR_1.

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 PAR_2 and PAR_4 and the disarming/inhibition of PAR_1. 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

of PAR_1, PAR_2, and PAR_4; 2) PAR-dependent calcium signaling responses in cells expressing PAR_1, PAR_2, and PAR_4, and in human platelets; 3) a vascular ring vasorelaxation assay using rat and murine tissue as well as murine tissue from PAR_2 null mice; and 4) a PAR_4-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 PAR_2 (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 G_q also implies a potential activation of G_i and other downstream signaling events, like the stimulation of mitogen-activated protein kinase, known to be regulated via PAR_1 and PAR_2.

Our focus was principally on PAR_2, 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 PAR_2. However, the actions of hK5 and -6 were distinct from those of hK14 in terms of their inability to activate PAR_4. The lack of activation of PAR_4 by these two kallikreins is in keeping with the very minor cleavage by hK5 and -6 we observed by HPLC analysis for the PAR_4-derived peptides and the lack of hydrolysis by hK6 of a model PAR_4 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 PAR_2 in many settings, adding to the ability of other serine proteinases like trypsin, factor VIIa/Xa, and matriptase to activate PAR_2. Unlike human mast cell tryptase, another potential physiological activator of PAR_2, 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 tryptase (58), hK14 was able to reduce vascular tension via PAR_2 activation in an intact rat and murine aorta tissue assay. That PAR_2 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 PAR_2 in which the tryptic cleavage/activation site was changed to a bond not susceptible to tryptic cleavage (Fig. 3*A*, tracing *C*); 2) by the lack of a relaxation response of endothelium-intact aorta rings derived from PAR_2 null mice that otherwise relaxed in response to PAR_1 activation (Fig. 9*A*, *tracings C* and *D*); and 3) by the disappearance of the N-terminal peptide sequence released by tryptic cleavage of rat PAR_2 (reduction of SLAW-A reactivity caused by hK14), with retention of the amino acid epitopes in the active tethered ligand sequence of rat PAR_2 recognized by the B5 antibody (Fig. 2). In this respect, hK5 and -6 yielded results comparable with those found for activation of PAR_2 by hK14. In the assay for PAR_2 activation in the KNRKrPAR$_2$ cells (Fig. 3*B,* 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 tryptase, with its restricted ability to activate PAR_2, the widely distributed tissue kallikreins via PAR_2 specifically and via PAR_1, PAR_2, and PAR_4 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 PAR_2 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 immunoassay. Such protein concentrations, if representative of active enzyme, would be sufficient to regulate the PARs.

Consequences of hK14 Disarming/Inhibition of PAR_1—The HPLC analysis of the hK14 cleavage products of hPAR_1 demonstrated a much lower degree of cleavage than for PAR_2 and PAR_4. Notwithstanding, hK14 hydrolysis of the PAR_1 peptide yielded about equal amounts of peptides resulting from cleavage at sites that would either activate or disarm the receptor (Fig. 1*A*). In the HEK cell and human platelet test systems, it was clear that hK14 preferentially disarmed/inhibited PAR_1 rather than causing activation. This result paralleled exactly our previous data obtained for the disarming of PAR_1 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 PAR_1-activating peptide, TFLLR-NH_2, pointed to a removal of the PAR_1 tethered ligand, as predicted by the peptide proteolysis data. This preferential disarming/inhibition of PAR_1 by hK14 is similar to the ability of neutrophil elastase to disarm/inhibit PAR_2 (16, 17). For cell regulation, the enzymatic disarming/inhibition of a PAR may be as important as its activation. By preferentially activating human platelet PAR_4 (Fig. 8) while disarming PAR_1 (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 PAR_4 *versus* PAR_1

mirrors the action of plasmin on human platelets, wherein plasmin can both activate and disarm PAR_1 (60, 61). Whether PAR_1 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 tryptic cleavage sites. Furthermore, by selectively activating PAR_2 and PAR_4, while disarming PAR_1, 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 nontryptic 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 $hPAR_1$ peptide at the SF/LL bond by hK14 that would disarm PAR_1 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 PAR_4—Human and rat PAR_4 also served as a target for hK14-mediated activation but not for hK5 and -6. The ability of hK14 to activate PAR_4 was demonstrated both by the rat platelet aggregation response to hK14, which is PAR_4-dependent (49), and by the calcium signaling responses in rat PAR_4-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 PAR_4 ($EC_{50} \cong 15$ units/ml; Fig. 7*B*) was comparable with but lower than its potency for activating either rat or human PAR_2 ($EC_{50} \cong 8$ units/ml; Figs. 3*B* and 4*B*). In the human platelet calcium signaling assay, hK14 was also able to activate PAR_4, as indicated by the calcium signal generated in the presence of a PAR_1 antagonist and from the data obtained using the PAR_4 desensitization protocol (Fig. 8). As already mentioned, neither hK5 nor hK6 was able to activate either rat or human PAR_4, 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 PAR_4.

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 PAR_1 and PAR_2 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 (G_q, G_i, and $G_{12/13}$), 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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