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Short Communication

Kallikrein-mediated cell signalling: targeting proteinase-activated receptors (PARs)*

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Abstract

We tested the hypothesis that human tissue kallikreins (hKs) may regulate signal transduction by cleaving and activating proteinase-activated receptors (PARs). We found that hK5, 6 and 14 cleaved PAR N-terminal peptide sequences representing the cleavage/activation motifs of human PAR_1 and PAR_2 to yield receptor-activating peptides. hK5, 6 and 14 activated calcium signalling in rat PAR_2-expressing (but not background) KNRK cells. Calcium signalling in HEK cells co-expressing human PAR_1 and PAR_2 was also triggered by hK14 (via PAR_1 and PAR_2) and hK6 (via PAR_2). In isolated rat platelets that do not express PAR_1, but signal *via* PAR_4, hK14 also activated PAR-dependent calcium signalling responses and triggered aggregation. The aggregation response elicited by hK14 was in contrast to the lack of aggregation triggered by hK5 and 6. hK14 also caused vasorelaxation in a phenylephrine-preconstricted rat aorta ring assay and triggered oedema in an *in vivo* model of murine paw inflammation. We propose that, like thrombin and trypsin, the kallikreins must now be considered as important 'hormonal' regulators of tissue function, very likely acting in part via PARs.

Keywords: inflammation; kallikreins; receptors; serine proteinases; signal transduction; trypsin.

In addition to their ability to generate active hormones from polypeptide precursors and to function as digestive enzymes, proteinases are now known to play a hormone-like role by triggering signal transduction pathways in target cells. In part, the physiological actions of proteinases such as thrombin and trypsin are mediated by 'proteinase-activated receptors' (PARs). The unique mechanism whereby serine proteinases such as trypsin and thrombin signal via the PARs involves proteolytic cleavage of the receptor N-terminal sequence to reveal an activating tethered ligand (Coughlin, 2000; Macfarlane et al., 2001; Hollenberg and Compton, 2002; Ossovskaya and Bunnett, 2004; Steinhoff et al., 2005). Like the tethered ligand, synthetic peptides derived from the revealed tethered ligand sequences can selectively activate PARs in the absence of proteolytic cleavage (Vu et al., 1991). For instance, TFLLR-NH_2 selectively activates PAR_1, whereas SLIGRL-NH_2 and AYPGKF-NH_2 selectively activate PAR_2 and PAR_4, respectively (Hollenberg et al., 1993, 1996, 1997, 2004; al-Ani et al., 1995, 2002; Saifeddine et al., 1996; Kawabata et al., 1999; Hollenberg and Saifeddine, 2001). PAR_1, PAR_3, and PAR_4 have been found to be targets for thrombin (Rasmussen et al., 1991; Vu et al., 1991; Coughlin, 2000), whereas PAR_2 is not readily activated by thrombin. Instead, PAR_2 can be activated by trypsin, tryptase and by other serine proteinase members of the clotting cascade apart from thrombin (e.g., the tissue factor VIIa-Xa complex; Nystedt et al., 1994; Ruf et al., 2003; Ossovskaya and Bunnett, 2004). Although pancreatic trypsin is known to activate intestinal epithelial PAR_2 (Kong et al., 1997), human mast cell tryptase activates PAR_2 only in selected settings (Mirza et al., 1997; Molino et al., 1997; Corvera et al., 1999; Compton et al., 2002a,b). In the PARs, these serine proteinases reveal the N-terminal tethered ligand by cleaving a target N-terminal arginine in the receptor sequence to unmask the tethered ligand (Vu et al., 1991). Except for the acknowledged physiological role of thrombin as an activator of PAR_1 and PAR_4, the true physiological activators of PAR_2 are essentially not known, except in the intestinal tract, where pancreatic trypsin is present in sufficient amounts to activate this receptor (Kong et al., 1997).

As possible physiological activators of the PARs, we have put forward the hypothesis that tissue kallikreins,

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Regarding a recommendation for future nomenclature of kallikrein gene-derived proteases, see the article 'A comprehensive nomenclature for serine proteases with homology to tissue kallikreins' by Lundwall et al., this issue pp. 637–641.

now known to comprise a large family of secreted serine proteinases with tryptic or chymotryptic activity, may represent important physiological regulators of the PARs (Borgono and Diamandis, 2004; Borgono et al., 2004). In humans, the kallikreins are abundantly expressed in groups in many tissues, often in a sex-steroid hormone-dependent manner. These enzymes have been observed to be upregulated in many types of cancer, both in the local tumour area and in the circulation. This situation is perhaps best known for the prostate-localised human kallikrein hK3, widely known as a cancer-associated 'prostate-specific antigen' (PSA). In addition, hK5, hK6 and hK14 are found at high levels in the serum and/or ascites fluid of ovarian cancer patients (Diamandis et al., 2000; Kim et al., 2001; Tanimoto et al., 2001; Borgono et al., 2003; Yousef et al., 2003). Kallikreins 5 and 14 are also elevated in the serum of breast cancer patients (Borgono et al., 2003; Yousef et al., 2003). Furthermore, kallikreins, such as kallikrein 6, are known to be highly expressed at sites of inflammation in the central nervous system (Blaber et al., 2002; Scarisbrick et al., 2002). In spite of these many settings in which kallikreins can be identified, the mechanisms by which this enzyme family regulates tissue function are not clear. Many studies carried out *in vitro* have identified proteins of the extracellular matrix, pro-urokinase-plasminogen activator (pro-uPA), kininogens, growth factor precursors (and binding proteins) and other kallikreins as potential targets of kallikrein proteolysis in the setting of cancer progression (Frenette et al., 1997; Takayama et al., 2001; Choong and Nadesapillai, 2003; Borgono and Diamandis, 2004; Borgono et al., 2004). Such substrates may well explain some but by no means all of the physiological actions of kallikreins, particularly in the setting of cancer. As already mentioned, we believe that the kallikreins, the majority of which exhibit potent trypsin-like serine proteinase activity, are good candidates to be considered as endogenous *in vivo* regulators of the PARs.

In view of the discussion in the previous paragraph, we tested the hypothesis that tissue kallikreins can act via the PARs using the following approaches: (1) an analysis using proteomic methodologies of the cleavage products yielded upon incubation of hK5, 6 and 14 with PAR N-terminal peptide sequences representing the cleavage/activation motifs of human PAR_1 and PAR_2; (2) a study of PAR-dependent calcium signalling responses (a) in cultured cells only expressing PAR_2, (b) in cultured cells expressing PAR_1 and PAR_2, and (c) in isolated rat platelets that do not express PAR_1, but signal via PAR_4; (3) a PAR_4-dependent rat platelet aggregation assay; and (4) a pharmacological approach (a) with a vascular ring vasorelaxation assay using tissues from both rats and from mice, and (b) with a murine paw oedema inflammation model. For our exploratory work described here, we used hK14 as a 'prototype' trypsin-like kallikrein because of its particularly wide tissue distribution. Furthermore, we focussed on the ability of hK14 to regulate PAR_2 in particular, because of the well-recognised susceptibility of this PAR family member to trypsin-like serine proteinase activation. Added to our focus on PAR_2, we evaluated hK14 for its ability to regulate PAR_1 and we determined whether hK5 and 6 might also be able to cleave peptides representing the tethered ligand sequences of human PAR_1 and PAR_2.

As outlined in Table 1, hK5, 6 and 14 were all able to cleave the synthetic tethered ligand sequences of human PAR_1 and PAR_2 at the key arginine to yield the PAR-activating sequences, SFLLRN… (for PAR_1) and SLIGKV… (for PAR_2). These data indicate that in intact human cells expressing PAR_1 and PAR_2, all three kallikreins could potentially trigger signals by revealing the active tethered ligands of the receptors. However, the cleavage data (Table 1) also showed that hK14 could cleave the synthetic tethered ligand sequence of human PAR_1 at positions downstream from the principal R/S cleavage/activation site (e.g., at the tryptic R/N and chymotryptic F/L target sequences in human PAR_1: SF/LLR/NPNDKYE…). Hydrolysis at these sites within or downstream from the tethered ligand sequence would, in intact cells, disconnect the activating tethered ligand SFLLRN sequence from its C-terminal tether, thereby disarming PAR_1 and preventing its activation by thrombin. Similarly, by cleaving at the K/V site in the tethered ligand sequence of human PAR_2, hK14 could in principle also disarm PAR_2 in intact cells. A comparable ability of hK5 and 6 either to reveal a tethered ligand activating sequence or to disarm PAR_1 and PAR_2 was also observed (Table 1). Equivalent data were obtained for the cleavage of the rat tethered ligand sequence representing rat PAR_2, indicating potentially either activation or disarming of rat PAR_2. Thus, in principle, the biochemical data supported the working hypothesis that hKs could activate PAR_1 and PAR_2; however, the data also indicated that, like trypsin (Kawabata et al., 1999), the hKs could potentially disarm one or both of PAR_1 and PAR_2.

Our next step was to test the ability of hK5, 6 and 14 to signal via PARs in intact cells. We focussed principally on the actions of hK14, comparing its activity with that of either hK6 or hK5, where appropriate. To monitor PAR-triggered calcium signalling, we turned to the use of: (1) a rat Kirsten murine sarcoma virus-transformed normal rat kidney (KNRK) cell line (KNRKrPAR_2) that expresses recombinant rat PAR_2, but not the other PARs, and for which non-transfected cells do not respond to tryptic or peptide PAR agonists (al-Ani et al., 2002); (2) a human embryonic kidney cell line (HEK) that constitutively expresses functional human PAR_1 and PAR_2 (Kawabata et al., 1999); and (3) a rat platelet suspension that is triggered by PAR_4 activation, but not via PAR_1 or PAR_2 (Hollenberg et al., 2004). The experimental approach that we used to monitor enzyme-mediated calcium signalling, as well as the cross-desensitisation protocols for establishing the PAR selectivity of PAR agonists, has been well documented (Kawabata et al., 1999). Using this type of assay, we have previously established that in PAR_2-expressing KNRK cells and HEK cells, calcium signals generated by proteolytic enzymes such as trypsin and thrombin result from PAR activation and not from other mechanisms (Hollenberg et al., 1997; Kawabata et al., 1999). For the cell signalling and tissue bioassay experiments (below), we elected to use concentrations of the kallikreins that were in keeping with the concentrations of trypsin we routinely use for our work (from approx. 0.5 to 30 U/ml). Since the conversion of zymogen to active

Table 1 Cleavage of synthetic PAR tethered ligand sequences by hKs 5, 6 and 14[a].

$hPAR_1$ (substrate: NATLDPRSFLLRNPNDKYE)	
Trypsin	NATLDPR—SFLLRNPNDKYE
hK14	NATLDPR—SFLLRNPNDKYE
	NATLDPRSF—LLRNPNDKYE
	NATLDPRSFLLR—NPNDKYE
hK5	NATLDPR—SFLLRNPNDKYE
	NATLDPRSF—LLRNPNDKYE
hK6	NATLDPR—SFLLRNPNDKYE
	NATLDPRSF—LLRNPNDKYE
	NATLDPRSFLL—RNPNDKYE
	NATLDPRS—FLLRNPNDKYE
	NATLDPRSFLLR—NPNDKYE
$hPAR_2$ (Substrate: acetyl-GTNRSSKGRSLIGKVDGTSHVTGKGVT-amide)	
Trypsin	GTNRSSKGR—SLIGKVDGTSHVTGKGVT
	GTNRSSKGR—SLIGKVDGTSHVTGK—GVT
	GTNRSSKGRSLIGK—VDGTSHVTGK—GVT
	GTNRSSKGRSLIGKV—DGTSHVTGK—GVT
hK14	GTNRSSKGR—SLIGKVDGTSHVTGKGVT
	GTNRSSKGRSLIGK—VDGTSHVTGKGVT
	GTNRSSKGRSLIGK—VDGTSHVTGK—GVT
	GTNRSSKGR—SLIGKVDGTSHVTGK—GVT
hK5	GTNRSSKGR—SLIGKVDGTSHVTGKGVT
	GTNR—SSKGRSLIGKVDGTSHVTGKGVT
hK6	GTNRSSKGR—SLIGKVDGTSHVTGKGVT
	GTNRSSKGRSLIGK—VDGTSHVTGK—GVT
	GTNRSSKGR—SLIGKVDGTSHVTGK—GVT

[a]The synthetic peptide substrates representing the cleavage/activation sequences of human PAR_1 and PAR_2 are shown in parentheses. The tethered ligand sequences in these substrate peptides are indicated by underlined letters. These substrates (100 μM) were incubated for 30 min at 37°C with the three human tissue kallikreins (hK5, 6 and 14; hK5, 4.5 U/ml; hK6, 1.2 U/ml; hK14, 6.2 U/ml) or trypsin (3.5 U/ml). The enzyme activities of all kallikrein preparations were standardised relative to the activity of trypsin (i.e., 'trypsin-like' U/ml) in terms of their ability to cleave specific proteinase substrates: Boc-Val-Pro-Arg-7-amino-4-methylcoumarin or Boc-Gln-Ala-Arg-7-amino-4-methylcoumarin. The activities of different preparations of enzyme varied, but the following molar approximations can be used for a 1 U/ml solution: hK5, 7 nM; hK6, 56 nM; hK14, 4 nM; trypsin, 2 nM. For hydrolysis of the synthetic tethered ligand sequences, the following buffers were used: 100 mM Na_2HPO_4, 0.01% Tween-20, pH 7.8 (hK5, hK14 and trypsin); 50 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, and 0.01% Tween-20, pH 7.3 (hK6). Reactions were terminated by rapid freezing in liquid nitrogen and samples were stored at -80°C. The cleavage products were identified by: (a) liquid chromatography-mass spectrometric analysis [Agilent HP 1100 Nanoflow System followed by linear ion trap quadrupole (LTQ); Thermoelectron, Norwood, NJ, USA] and/or (b) matrix-assisted laser desorption ionisation (MALDI) mass spectrometry at the Proteomics Facilities of Samuel Lunenfeld Research Institute (Mount Sinai Hospital, Toronto, Canada). The predicted cleavage sites in the tethered ligand sequences deduced from the mass spectral identity of the cleavage products are shown in the table by long spacers (—). Only the underlined sequences, resulting from proteolysis, that would remain tethered to the remainder of the cell-associated receptor would result in enzyme-mediated signalling. Other potential cleavage sites downstream from the tethered ligand sequence would disarm the receptor, preventing receptor activation.

enzyme can vary from one kallikrein preparation to another, each kallikrein sample used for our experiments was standardised in terms of its trypsin-equivalent activity (U/ml, as described in detail in the footnote to Table 1).

Each of kallikreins 5, 6 and 14 was able to trigger an elevation of intracellular calcium via PAR_2 in $KNRKrPAR_2$ cells (Figure 1). No calcium signal was observed in the background PAR_2 non-responsive cells (not shown). In KNRK cells expressing recombinant rat PAR_2 (but not in the background non-expressing KNRK cells), hK14 was also able to generate a calcium signal equivalent to that caused by the selective PAR_2-activating peptide, SLIGRL-NH_2 (Figure 2A). This activation of calcium signalling by hK14 in the PAR_2-expressing KNRK cells desensitised the cell response to subsequently added SLIGRL-NH_2 compared with the signal generated in the cells that had not been pre-exposed to hK14 (see arrow for comparison of the two SLIGRL-NH_2-generated calcium signals in Figure 2A). Like hK14, in PAR_2-expressing KNRK cells (but not in non-expressing cells), hK6 also stimulated elevations of intracellular calcium (Figure 2B). This activation of KNRK cells with hK6 desensitised the cell response to trypsin (see arrow comparing the trypsin signal in the left-hand tracing in Figure 2B, with the subsequent tracing shown for trypsin action alone). Furthermore, after pre-exposing PAR_2-expressing cells to hK6, the subsequent response to the PAR_2 agonist, SLIGRL-NH_2, was markedly diminished (right-hand portion of last tracing in Figure 2B) compared to the response of cells that had not been pre-exposed to hK6 (control response to SLIGRL-NH_2, second-last tracing on right in Figure 2B). The cross-desensitisation by hK14 of the response triggered by SLIGRL-NH_2 and the ability of hK6 to desensitise signalling in PAR_2-expressing KNRK cells by both trypsin and SLIGRL-NH_2 clearly demonstrates the ability of hK14 and hK6 to activate PAR_2 (Kawabata et al., 1999).

As shown in Figure 3A, hK14 was also able to generate a calcium signal in HEK cells that was somewhat larger

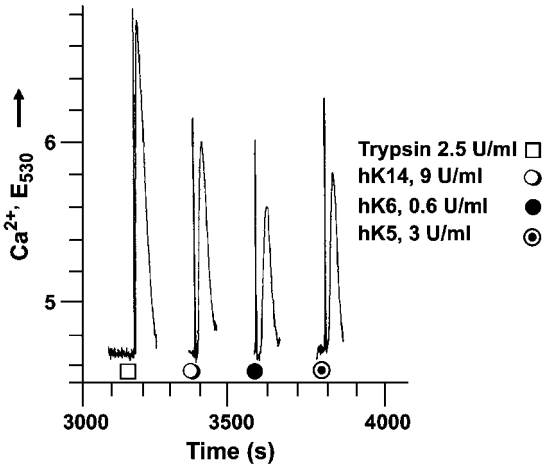

Figure 1 Activation of calcium signalling by hK5, 6 and 14 via PAR_2 in rat PAR_2-expressing KNRK cells.
KNRK cells expressing rat PAR_2 ($KNRKrPAR_2$; Al-Ani et al., 2002) were exposed to the concentrations of hK5, 6 and 14 indicated for measurements of calcium signalling (Kawabata et al., 1999) and the responses were compared to that triggered by trypsin (right-hand tracing). Time (s) is shown on the X-axis; elevation of intracellular calcium (E_{530}) is denoted by an upward deflection of the fluorescence tracing, shown in arbitrary fluorescence units on the Y-axis. Data represent comparable tracings observed in three or more independent experiments with individually grown $KNRKrPAR_2$ cell samples.

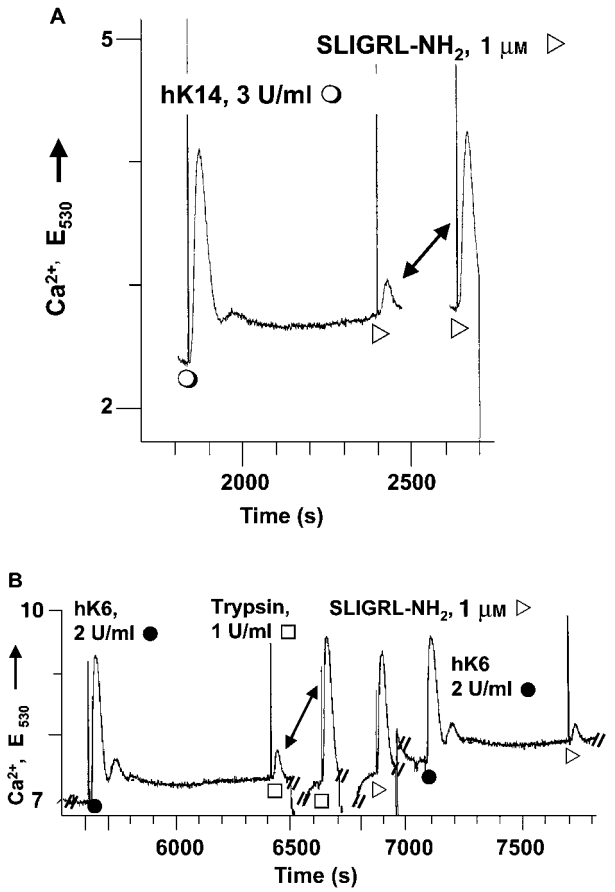

Figure 2 Calcium signalling triggered by hK14 and hK6 in rat KNRK cells expressing recombinant rat PAR_2.
(A) hK14 signalling cross-desensitises the receptor to activation by SLIGRL-NH_2. (B) hK6 signalling desensitises the receptor to activation by either trypsin or SLIGRL-NH_2. Using methods previously described in detail (Kawabata et al., 1999), cells loaded with the calcium indicator, Fluo-3, were exposed to hK14 (○, 3 U/ml) or hK6 (●, 2 U/ml), followed by the addition of either SLIGRL-NH_2 (▷, 1 μM) or trypsin (□, 1 U/ml: lower panel B only) to the stirred cell suspension. In separate cell suspensions (separated by the break symbol: //), signalling by either trypsin alone (□, 1 U/ml) or by the receptor-selective PAR_2-activating peptide SLIGRL-NH_2 (▷, 1 μM) alone (last tracing on the right in panel A; second-last tracing on the right in panel B) was monitored. The diminished response to SLIGRL-NH_2 and trypsin (data shown only for hK6) after pre-treatment of the cells with either hK14 or hK6 (arrows point to signals before and after enzyme exposure) indicated that both hK14 and hK6 were signalling via PAR_2. Time (s) is shown on the X-axis; elevation of intracellular calcium (Ca^{2+}, E_{530}) is denoted by an upward deflection of the fluorescence tracing, shown in arbitrary fluorescence units on the Y-axis. Under these conditions, the values calculated for intracellular calcium in the HEK and KNRK cells were approximately 30 nM under basal conditions and maximally approx. 340 nM upon exposure to SLIGRL-NH_2 (Kawabata et al., 1999). Data represent comparable tracings observed in three or more independent experiments with individually grown rat PAR_2-expressing KNRK cell samples. KNRK cells that did not express recombinant PAR_2 did not yield a calcium signal in response to hK6, hK14 or SLIGRL-NH_2 (not shown).

in magnitude than that generated by thrombin alone acting at its EC_{50} (right-hand tracing, Figure 3A). This signal was presumed to result from the combined activation of PAR_1 and PAR_2 by hK14. Subsequent to the activation of HEK cells by hK14, the signal caused by thrombin was considerably diminished compared to the control thrombin signal generated in cells that had not been pre-exposed to hK14 (see arrow for comparison with the signal shown on the far right of the tracing in Figure 3A). This result indicates that the first exposure of the cells to hK14 led to activation-desensitisation and/or disarming of PAR_1 in HEK cells. Thus, the subsequent response to thrombin was attenuated. Similarly, pre-treatment of HEK cells with an activating concentration of hK14 desensitised the cell response to the PAR_2 agonist, SLIGRL-NH_2 (right-hand portion of first tracing in Figure 3B) compared with the signal caused by the peptide in cells that were not pre-exposed to hK14 (see arrow for comparison of the two SLIGRL-NH_2-triggered signals in Figure 3B).

Data obtained from the calcium signalling experiments using KNRKrPAR$_2$ and HEK cells thus indicate that hK14 is capable of activating/desensitising both PAR_1 and PAR_2. The preliminary study showed that like hK14, hK5 and hK6 were also able to activate PAR_2, but more work using HEK cells is required to assess the impact of hK5 and hK6 on PAR_1. Further detailed experiments are

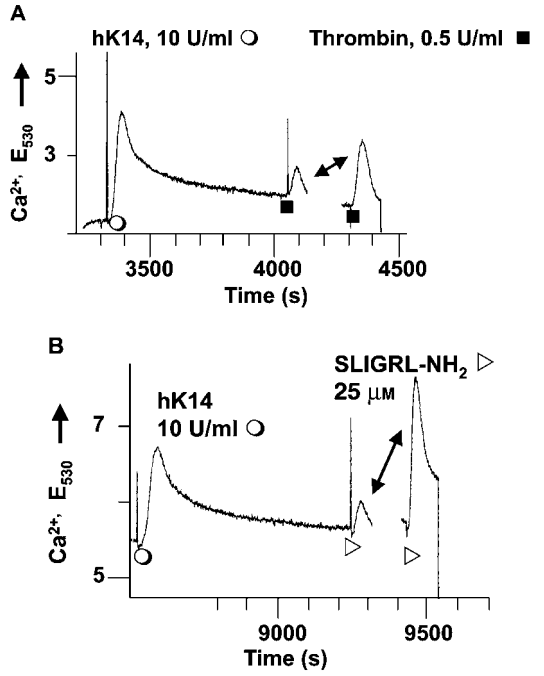

Figure 3 Calcium signalling triggered by hK14 in cultured human embryonic kidney cells (HEK) co-expressing functional PAR_1 and PAR_2.
(A) Signalling by hK14 desensitises the signal triggered by thrombin. (B) Signalling by hK14 desensitises the signal generated by the PAR_2-selective agonist, SLIGRL-NH_2. The arrows show a comparison of the calcium signals generated by either thrombin or SLIGRL-NH_2 either after (left) or before (right tracings) the cells were exposed to hK14. As described in detail previously for measuring PAR-mediated calcium signalling (Kawabata et al., 1999), cells loaded with the calcium indicator, Fluo-3, were exposed to hK14 (○, 10 U/ml), followed by the addition of thrombin (■, 0.5 U/ml) to the cuvette. A separate cell suspension was exposed to the same concentration of thrombin without prior exposure to hK14 (right-hand tracing). Time (s) is shown on the X-axis; elevation of intracellular calcium (E_{530}) is denoted by an upward deflection of the fluorescence tracing, shown in arbitrary fluorescence units on the Y-axis. Data represent comparable tracings observed in three or more independent experiments with individually grown HEK cell samples.

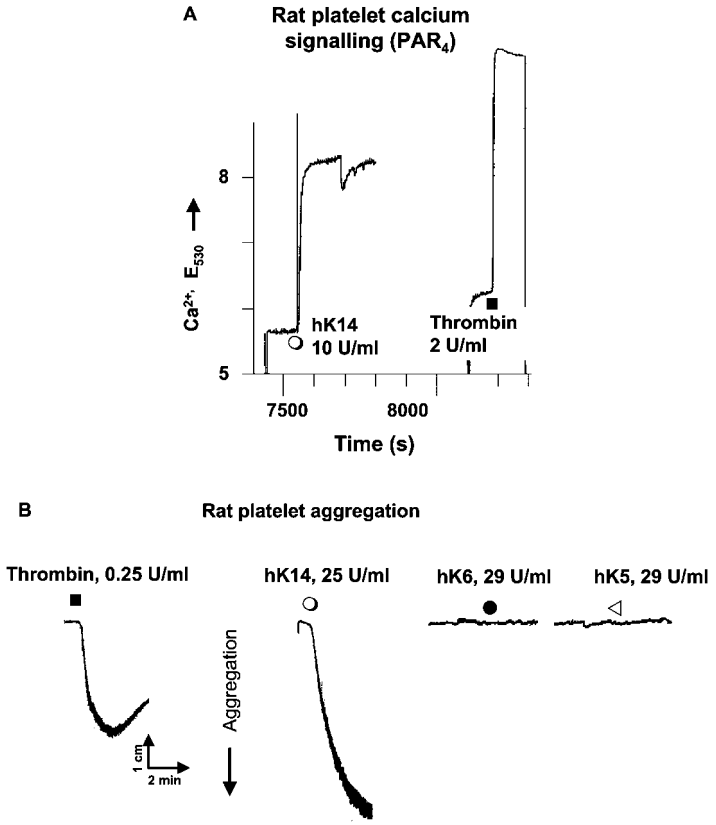

Figure 4 Activation of rat platelet PAR_4 by hK14, but not by hK6 and 5: calcium signalling (A) and aggregation (B).
Washed rat platelets were isolated from male Sprague Dawley rats as previously described (Hollenberg et al., 2004) and were either loaded with Fluo-3 for calcium signalling experiments (A), as for the HEK or KNRK cells, or were used for a platelet aggregation assay (B), as also described previously (Hollenberg et al., 2004). The responses to hK14 and the lack of aggregation response to hKs 6 and 5 were compared with the responses to thrombin. The data are representative of four (A) or six (B) independently conducted experiments with different platelet preparations. Increased intracellular calcium (Ca^{2+}, E_{530}) is shown (A) in arbitrary fluorescence units on the Y-axis; aggregation (B) is indicated by the downward deflection of the turbidity (arrow).

therefore warranted to determine the precise action of the kallikreins on these two PARs.

As an index of the ability of hKs to activate PAR_4, we made use of a rat platelet calcium signalling assay and a rat platelet aggregation assay, in which the response to trypsin and thrombin is entirely due to the activation of PAR_4 and not PAR_1, since the rat platelet does not express PAR_1 (Hollenberg and Saifeddine, 2001; Hollenberg et al., 2004). In the rat platelet calcium signalling assay, hK14 (10 U/ml) was able to cause a prompt platelet response that was approximately 60% of that caused by maximally active amounts of thrombin (2 U/ml) (Figure 4A). In the rat platelet aggregation assay, which is PAR_4-dependent in terms of the thrombin response, hK14 (25 U/ml), like thrombin (0.25 U/ml), triggered an aggregation response (Figure 4B). However, at equivalent enzyme concentrations, neither hK5 nor hK6 was able to cause rat platelet aggregation (right-hand traces, Figure 4B). Thus, in rat platelets, hK14 appears to signal like thrombin, presumably via PAR_4, and neither hK5 nor hK6 appears to activate this receptor.

In previous work, we established the ability of trypsin to cause an endothelium-dependent, nitric oxide-mediated relaxation via PAR_2 activation (Hollenberg et al., 1996, 1997). We used the same vascular bioassay approach (Hollenberg et al., 1996, 1997) to evaluate the action of hK14. As shown in Figure 5A, in an endothelium-intact rat aorta preparation pre-constricted with 1 μM phenylephrine, hK14 (21 U/ml) was able to cause a relaxation response equivalent to that of 10 μM acetylcholine. Following prior exposure to hK14, the relaxant response of the tissue preparation to trypsin (24% of the relaxant action of 10 μM acetylcholine) was diminished compared to the control relaxant response to trypsin observed in the absence of hK14 pre-treatment (75% of the relaxant action of 10 μM acetylcholine; Figure 5B). These data indicate that, like trypsin, hK14 has vasoregulatory properties, very likely due to the activation of endothelial cell PAR_2 (Hollenberg et al., 1997). Further work with aorta tissue derived from $PAR_2^{-/-}$ mice is required to substantiate the role of PAR_2 in the regulation of vascular tension by kallikreins.

It has already been established that trypsin, acting via PAR_2, can cause an inflammatory response, as monitored in a paw oedema model of inflammation and a model of colitis (Vergnolle et al., 1999b, 2001b; Cenac et al., 2002, Nguyen et al., 2003). We hypothesised that hK14 could mimic the action of trypsin in a murine paw oedema inflammation model. Thus, with the methodology previously employed to assess the inflammatory action of agonists of PAR_4 (Hollenberg et al., 2004), we used the murine paw oedema model to examine the potential inflammatory action of hK14 via PARs (Figure 6). Indeed, intra-plantar administration of hK14 (18 U/paw) caused

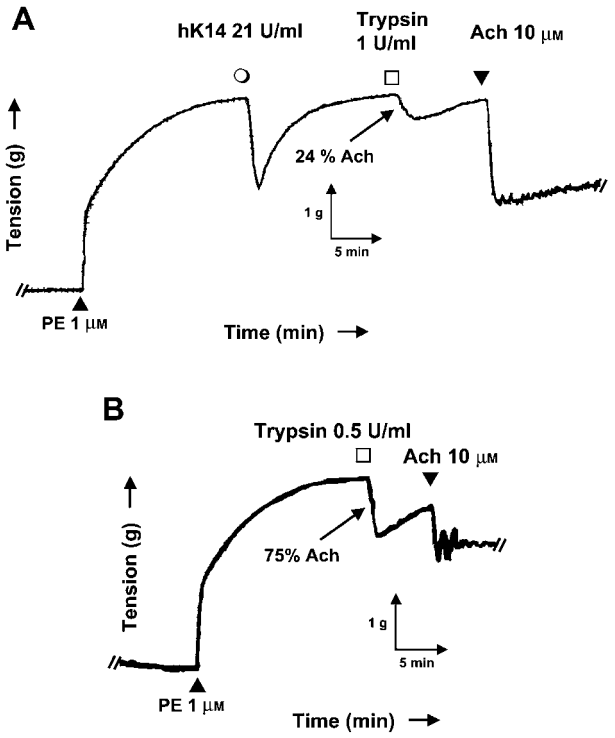

Figure 5 hK14-mediated vasorelaxation in an endothelium-intact rat aorta ring preparation.
(A) An endothelium-intact rat aorta ring preparation (responsive to 10 μM acetylcholine: ▼, Ach) was pre-constricted with phenylephrine (▲, 1 μM PE) and allowed to reach a plateau of tension, using methods previously described in detail (Hollenberg et al., 1993, 1996, 1997, 2004). The preparation was then exposed to hK14 (○, 21 U/ml) while continually monitoring the tissue tension. After the transient relaxation caused by hK14 had returned towards maximum tension, trypsin (□, 1 U/ml) was added to the organ bath to monitor its relaxant effect after pre-exposure of the tissue to hK14. The relaxant responses of the tissue to hK14 and trypsin were compared with the relaxant effect of acetylcholine (▼, 10 μM Ach). (B) Without pre-treatment of the tissue with hK14, the relaxant effect of trypsin was approximately 75% of the relaxation caused by 10 μM acetylcholine (Ach), whereas the response to trypsin after pre-treatment with hK14 was only approximately 24% of the relaxant response to 10 μM acetylcholine in the same tissue. The scales for time (min, X-axis) and tension (g, Y-axis) are shown by the insets. The data are representative of three independently conducted experiments.

an oedema response in mice (increased paw diameter in mm), with an increase in diameter over baseline of 0.37±0.12 mm (average increase in diameter ±SEM, n=5) at 30 min. The swelling was maintained at 60 min (0.50±0.13 mm). The increase in oedema caused by hK14 (Figure 6) was in keeping with the oedema caused by trypsin over the same time course (data not shown). Administration of the same amount of heat-inactivated hK14 resulted in little oedema at the 30-min (0.024±0.01 mm) and 60-min time points (0.050±0.01 mm). This lack of swelling was in stark contrast ($p<0.005$) to the persistent oedema caused by active hK14. The pattern of oedema response over time was the same as that previously shown for PAR-induced oedema (Hollenberg et al., 2004). We conclude that, as for trypsin and tryptase, hK14 can cause an inflammatory response in murine paw. As suggested by our *in vitro* data showing PAR activation by hK14, and the large volume of literature

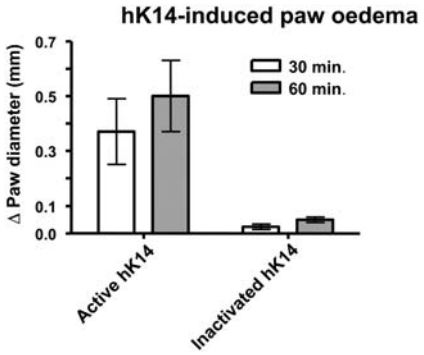

Figure 6 hK14-induced murine paw oedema.
The increased paw oedema (Δ, mm) in response to hK14 administration was measured as outlined in the text at 30 and 60 min for either active (group 1: left histograms) or heat-inactivated hK14 (group 2: right histograms) (18 U/paw). The bars in the histograms represent the average increases (Δ, mm±SEM, n=5 for groups 1 and 2) in paw diameter.

data demonstrating a pro-inflammatory role for PAR activation (Steinhoff et al., 2005; Vergnolle, 2005), this pro-inflammatory effect of hK14 could be mediated by PAR activation.

Our data demonstrate that human tissue kallikreins (hK5, 6 and 14) can cleave synthetic PAR-related tethered ligand peptide sequences in a manner that in intact cells could potentially result in either the generation of PAR-activating tethered ligand sequences or in the disarming of PAR_1 and PAR_2. Furthermore, hK6 and 14 both mimic PAR_2 cellular signalling caused by PAR_2-activating peptides and by trypsin (elevation of intracellular calcium; endothelium-dependent vasorelaxation). The cross-desensitisation experiments using HEK cells and the PAR_2-expressing KNRK cell line conclusively demonstrated activation of PAR_2 by hK6 and 14. These results are consistent with the ability of hK14 to cause a trypsin-like oedema response in the murine paw inflammation model. Our data also indicate that in addition to PAR_2, PAR_1 and PAR_4 also appear to be targets for kallikrein regulation. The rat platelet aggregation and calcium signalling results strongly support our hypothesis that hK14 can activate PAR_4. Furthermore, the platelet aggregation data show that there can be a differential response of tissues to hK14 compared to hK5 and hK6.

In summary, our data add PARs to the list of kallikrein targets that may explain the signalling properties that this enzyme family has in many tissues. By activating the PARs, the kallikreins can potentially signal by a variety of G protein-coupled signalling pathways (G_q, G_i, $G_{12/13}$) (Macfarlane et al., 2001), thus regulating processes ranging from cell migration, inflammation and nociception to tumour angiogenesis, growth, metastasis and invasion. The effects that kallikreins may have in any situation may depend on the spectrum of kallikreins present and the availability of PARs that may be expressed in any specific tissue. We suggest that kallikreins very likely represent widely distributed endogenous PAR regulators that may be as important as thrombin in terms of their biological impact by activating the PARs. Apart from these receptor-mediated signalling pathways, serine proteinases such as the kallikreins can generate novel receptor-activating agonists and can potentially regulate signalling

pathways via non-receptor mechanisms (e.g., modulating matrix-integrin interactions), which can be added to their recognised ability to generate active polypeptides from pro-agonist protein precursors. The signalling properties of the kallikreins via the PARs add a novel dimension to the biological significance of this enzyme superfamily. Based on our previous findings related to the potential pathophysiological roles of the PARs (Vergnolle et al., 1999a, 2001a,b; Noorbakhsh et al., 2003, 2005; Vergnolle, 2004), we propose that the kallikreins, via PAR-mediated signalling, may play a prominent role in a wide variety of pathologies, including cancer. 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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