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Endothelium-dependent contractile actions of proteinase-activated receptor-2-activating peptides in human umbilical vein: release of a contracting factor *via* a novel receptor

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1 The contractile actions of the proteinase-activated receptor-2-activating peptides (PAR_2APs), SLIGRL-NH_2 (SL-NH_2), SLIGKV-NH_2 (KV-NH_2), trans-cinnamoyl-LIGRLO-NH_2 (tc-NH_2), and the PAR_1-AP, TFLLR-NH_2 (TF-NH_2) as well as trypsin and thrombin were studied in endothelium-denuded and intact human umbilical vein (HUV) ring preparations.

2 In HUV rings with, but not without an intact endothelium, PAR_2APs caused a concentration-dependent contractile response, whereas LSIGRL-NH_2 trypsin and PAR_1APs were inactive. The contractile response was not affected by the endothelin ET_A receptor antagonist, BQ123, the cyclooxygenase inhibitor, indomethacin, the leukotriene synthesis inhibitor, MK886, or the epoxygenase/P450 inhibitor, SKF-525A. Other pharmacological antagonists (prazosin, Losartan®) were similarly inactive.

3 The order of potencies of the PAR_2APs to cause a contraction in the endothelium-intact preparation was: SL-NH_2 > > KV-NH_2 ≥ tc-NH_2.

4 Using an endothelium-free rat aorta ring as a reporter tissue, surrounded with endothelium-intact HUV as a donor tissue in a 'sandwich assay,' we also monitored the ability of SL-NH_2, TF-NH_2, trypsin and thrombin to release either contractile (EDCF) or relaxant (EDRF) factors.

5 In the 'sandwich assay' done in the presence of L-NAME (0.1 mM), the endothelium-intact HUV tissue (but not endothelium-denuded HUV) released a contractile factor (EDCF) in response to SL-NH_2 (50 μM) but not to trypsin or LSIGRL-NH_2. The SL-NH_2-mediated release/action of the EDCF was not affected by BQ123, indomethacin, MK886 or SKF-525A.

6 In the 'sandwich assay', trypsin (4–10 nM), SL-NH_2, KV-NH_2 and tc-NH_2 caused the release of a relaxant activity (EDRF) from the endothelium-intact (but not the denuded) HUV preparation. The release of EDRF was blocked by 0.1 mM $^{\omega}$nitro-L-arginine-methylester (L-NAME). Neither thrombin (10 u ml^{-1}, 100 nM) nor TF-NH_2 (50 μM) were active in this EDRF-release assay.

7 The relative potencies of the PAR_2 agonists for causing the release of EDRF in the HUV sandwich assay were: trypsin > > SL-NH_2 > > tc-NH_2 > KV-NH_2. This order of potencies differed from the one observed for the same agonists in the HUV contraction assay (above) and in an intracellular calcium signalling assay, conducted with cloned human PAR_2 that was expressed in cultured rat kidney KNRK cells: trypsin > > SL-NH_2 = tc-NH_2 > KV-NH_2.

8 We conclude that PAR_2APs (but not PAR_1APs) *via* a receptor distinct from PAR_2, can cause a contractile response in endothelium-intact HUV tissue *via* the release of a diffusable EDCF, that is different from previously recognized smooth muscle agonists (e.g. prostanoid metabolites, endothelin, noradrenaline, angiotensin-II, acetylcholine).

Keywords: Proteinase-activated receptor; PAR_2; PAR_1; umbilical vein; endothelium-derived contracting factor; trypsin; thrombin

Introduction

The proteinases, thrombin and trypsin, apart from their ability to initiate proteolytic enzyme cascades, are now known to regulate cellular function by the stimulation of G-protein-coupled 'tethered-ligand' receptors. The unique mechanism for the activation of these so-called proteinase-activated receptors (PARs) involves the proteolytic unmasking of an N-terminal sequence that stays attached to the remainder of the 7-transmembrane receptor and acts as a 'tethered' activating ligand (Vu *et al*., 1991; Coughlin *et al*., 1992). To date, four members of this novel receptor family have been described, three of which can be activated preferentially by thrombin (PAR_1, PAR_3 and PAR_4; Vu *et al*., 1991; Rasmussen *et al*., 1991; Ishihara *et al*., 1997; Xu *et al*., 1998; Kahn *et al*., 1998) and one of which (PAR_2) can be activated by either trypsin (Nystedt *et al*., 1994; 1995a, b) or tryptases (Corvera *et al*., 1997; Molino *et al*., 1997; Mirza *et al*., 1997). A remarkable property of PAR_1 and PAR_2 (but not PAR_3) is that in the absence of proteolytic cleavage, these receptors can be activated by exposure to relatively short peptides based on the sequences of the 'revealed' tethered ligand e.g. SFLLRNP... for human PAR_1 (Vu *et al*., 1991) or SLIGRL-NH_2 for rat and mouse PAR_2 (Nystedt *et al*., 1994; 1995a, b; Al-Ani *et al*., 1995; Saifeddine *et al*., 1996). In our own work, we have developed a panel of PAR-activating peptides (PAR-APs)

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that are relatively selective for activating either PAR_1 (e.g. TFLLR-NH_2 or TF-NH_2: Blackhart *et al*., 1996; Hollenberg *et al*., 1997) or PAR_2 (e.g. SLIGRL-NH_2: Blackhart *et al*., 1996; Hollenberg *et al*., 1997). We have used a number of such PAR-APs as surrogate receptor activators in lieu of the proteinases, in order to assess the pharmacologic consequences of PAR activation in a variety of tissues. Since PAR-antagonists are not yet available, it has been our working hypothesis, in keeping with the approach pioneered by Ahlquist (1948) for the identification of α and β adrenoreceptor systems, that structure-activity relationships (SARs) for a series of PAR-APs can serve to identify novel tethered-ligand receptor systems in a variety of target tissues. This approach has enabled us to establish, along with molecular biological techniques, the ability of concurrently expressed endothelial PAR_1 and PAR_2 receptors to regulate vascular contractility (Al-Ani *et al*., 1995; Saifeddine *et al*., 1996; Hollenberg *et al*., 1996; 1997). This same approach has also enabled us to identify in the rat vascular endothelium, a PAR_2AP-stimulated receptor system that causes vasoconstriction (Roy *et al*., 1998). In our previous work with human umbilical cord blood vessels (Tay-Uyboco *et al*., 1995), we had observed that PAR_1APs caused a contractile response only in tissue that had been stripped of its endothelium. Further, we were not able to monitor a relaxant response to PAR_1APs in an endothelium-intact HUV preparation (Tay-Uyboco *et al*., 1995). In view of our findings with the rat vasculature, wherein PAR_2APs were found to cause both endothelium-dependent relaxant and endothelium-dependent contractile responses (Roy *et al*., 1998), we wished to examine the human umbilical vein tissue in further depth, with two main questions in mind: (1) Given that functional receptors for both PAR_1 and PAR_2 had been detected in cultured umbilical vein-derived human endothelial cells (e.g. Mirza *et al*., 1996), could such endothelial cell receptors regulate muscle tension in endothelium-intact HUV tissue? and (2) given that endothelial cell nitric oxide synthase (eNOS) had been detected in HUV tissue by immunohistochemical methods (Buttery *et al*., 1994 and by a pharmacologic approach (Chaudhuri *et al*., 1991), might activation of PAR_1 or PAR_2 either enzymatically or with the use of PAR-APs in endothelium-intact HUV tissue result in the generation of nitric oxide (NO)? To answer these questions, we studied further in both endothelial-intact and endothelium-denuded HUV rings, the contractile activities of thrombin, trypsin, the PAR_1-selective activating peptide, TFLLR-NH_2 (TF-NH_2 Hollenberg *et al*., 1997), and the PAR_2-selective activating peptides SLIGRL-NH_2 (SL-NH_2), SLIGKV-NH_2 (KV-NH_2) and trans-cinnamoyl-LIGRL0-NH_2 (tc-NH_2) (Roy *et al*., 1998). Further, since in preliminary work we had determined that the HUV tissue *per se* is very resistant to the relaxant actions of NO (either 10 μM sodium nitroprusside or 1 μM NO itself) we developed a 'sandwich' assay, using an endothelium-denuded rat aorta reporter tissue, to detect the release of vasoregulatory substances from the endothelium-intact HUV donor tissues (e.g. see Furchgott & Zawadzki, 1980). Using the sandwich assay, we evaluated the ability of the same agonists employed in the contractile assays to release agents that might affect the contractility of the rat aorta reporter tissue. In addition, we compared the SAR relationships for the PAR_2APs in the HUV contraction and 'sandwich' assays with their relative potencies in a calcium signalling assay done with human PAR_2-expressing cultured receptor-transfected rat kidney KNRK cells.

Methods

Preparation of HUV tissue and contractile bioassay procedures

Full term human placental tissue with the attached umbilical cord was obtained from normal vaginal deliveries, through the courtesy of the Maternity Care Centre of the Foothills Hospital, Calgary, AB, Canada. Tissue was transported to the laboratory on ice and the HUV tissue was immediately dissected out from the middle 1/3 of the umbilical cord, to obtain vessels that were free from blood clots. HUV tissue (about 8 cm long) was cleaned free of Wharton's jelly and placed in ice-cold gassed (95% O_2/5% CO_2) Krebs-Henseleit buffer, pH 7.4, of the following composition (mM): NaCl 115, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10. Tissues (approx 0.5×8 cm vessels) were then either stored in saline at 4°C overnight prior to use or were immediately cut into rings (approx. 8 mm long $\times$ 5 mm diameter) and mounted in a thermostatted (37°C) plastic organ bath (4 ml volume) under 2 g tension in the buffer described above, gassed with 95% O_2/5% CO_2 to maintain the pH at 7.4. The responsiveness to all agonists of stored and freshly used tissues was comparable, but the contractile response of tissues stored overnight at 4°C was improved by supplementing the above described storage buffer with 50 mM KCl. Rings were either mounted intact, or were stripped of endothelium by rolling the tissue lumen over the end of a fine forceps. Placental HUV rings (either freshly prepared; or cut from tissue that had been stored at 4°C overnight) were allowed to equilibrate under a tension of 2 g for 2–5 h at 37°C in order to stabilize baseline tension. Tension was monitored isometrically with either Statham or Grass force-displacement transducers. The contractile integrity of each preparation was routinely evaluated by challenging the tissue with 50 mM KCl, which caused a contractile response on average of 2.0 ± 0.3 g (mean $\pm$ s.e.mean for $n = 19$). The contractile response of each tissue to 50 mM KCl was used as a reference standard for normalizing the contractile responses of different preparations to the PAR-APs, thrombin and trypsin. For each agonist, tension was allowed to reach a plateau, at which point the preparation was washed free of agonist (W, tissue wash in Figures) and allowed to return to baseline tension (about 50 min of re-equilibration before a subsequent challenge with agonist). This protocol resulted in reproducible responses to repeated additions of the agonists.

Contractions were expressed as a percentage (% KCl) of the 50 mM-KCl-induced contraction. Concentration-response curves for all agonists were obtained by measuring responses at each agonist concentration (relative to 50 mM KCl) with multiple tissue strips from over 20 individual tissue donors. Values shown in the concentration-response curves represent the mean values $\pm$ s.e.mean. (bars) for measurements done with more than seven individual ring preparations for each agonist concentration shown. When present, pharmacological antagonists [e.g. indomethacin, prazosin, Losartan® (DuP753)] were added to the organ bath 15 to 20 min prior to the addition of contractile agonist.

'Sandwich' assay

The 'sandwich' assay, used to detect the release of either relaxant or contractile activity from the HUV tissue, employed an endothelium-free ring of rat aorta tissue as a reporter tissue attached to the force-transducer, surrounded by a sleeve of HUV tissue that was pulled over the aorta ring (see Figure 1).

Endothelium-free rat aorta rings (about 5 mm long × 3 mm diameter) were prepared essentially as described previously (Roy *et al*., 1998; Laniyonu & Hollenberg, 1995) from heparinized male albino Sprague-Dawley rats (200–250 g) that were cared for in accordance with the guidelines of the Canadian Council on Animal Care and that were sacrificed by cervical dislocation after diethyl ether anaesthesia. Each 'reporter ring' was stripped of its endothelium by rolling over the end of a sharp forceps and was then equilibrated for about 1 h in the above-described gassed (95% O_2/5% CO_2) Krebs-Henseleit buffer at 37°C under a tension of 1 g. The integrity of each endothelium-denuded aorta ring reporter tissue was assessed by observing contractions caused by 50 mM KCl and 1 μM phenylephrine; and the absence of a functional endothelium in the reporter tissue was confirmed by the absence of relaxation upon adding 1 μM acetylcholine (Ach) to a preparation that had been precontracted with 1 μM phenylephrine. Once validated for use as a 'reporter tissue,' as evidenced by a robust contraction in response to 50 mM KCl and phenylephrine and a lack of relaxation in the presence of 1 μM Ach, the recording of tissue tension was interrupted, and the aorta ring was surrounded with a sleeve of HUV tissue (about 3 mm long × 5 mm diameter either intact or denuded of endothelium) before reconnecting the aorta reporter tissue to the strain gauge (Figure 1). To monitor the presence of an intact endothelium in the HUV tissue, the aorta reporter tissue was first precontracted with 1 μM phenylephrine, and 20 μM histamine was added to the organ bath at the plateau of the phenylephrine-induced contractile response; a relaxation caused by the addition of histamine was taken to reflect the integrity of the HUV endothelium, and the release of EDRF. After monitoring the response of the precontracted aorta tissue to the addition of histamine or the PAR-APs, the preparation was washed (W. arrows in Figures) re-equilibrated with fresh buffer and allowed to return to baseline tension. Tissues were challenged with agonists at about 60 min intervals. This time interval resulted in reproducible responses to a given concentration of agonist. The relaxant activity caused by the addition of PAR_2APs or trypsin was expressed as a percentage (% HIST) of the relaxation caused in the preparation by 20 μM histamine (HIST). Values for the concentration-effect curves for the ability of trypsin and the PAR_2APs to cause the release of relaxant activity from the HUV tissue represent the means ± s.e.mean for measurements done with 5 to 10 individual sandwich preparations for each concentration of agonist. To monitor the release of contractile activity from the HUV tissue, sandwich preparations were pretested and assembled as outlined above, but 0.1 mM N^{ω}-nitro-L-arginine methyl ester (L-NAME) was added to the organ bath 20 min prior to the addition of agonist (trypsin, PAR_2APs etc.) to inhibit endothelial nitric oxide synthase. The aorta tissue was then maintained at baseline tension (about 1 g), and an increase in tension of the aorta reporter tissue was taken as evidence for the release of a contractile substance from the HUV tissue, upon adding the PAR_2APs to the organ bath.

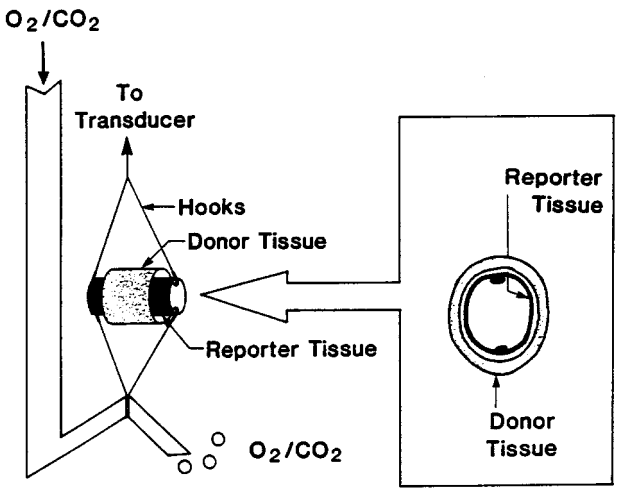

Figure 1 Schematic representation of the 'sandwich' assay using an endothelium-denuded rat aorta reporter tissue. Endothelium-denuded rat aorta tissue (inner tissue, solid circle) was first tested for its response to 50 mM KCl and 1 μM phenylephrine and its lack of response to 1 μM acetylcholine. Subsequently, the reporter tissue, attached to the strain gauge, was surrounded with HUV tissue (grey, outer circle) that was either intact or denuded of endothelium. Agonists were added directly to the organ bath and the tension in the aorta reporter tissue was monitored.

Preparation of human PAR_2-KNRK cell line and measurement of intracellular calcium signals

The human PAR_2 (Böhm *et al*., 1996) subcloned into pcDNA3 (In Vitrogen, San Diego, CA, U.S.A.) was expressed in cloned receptor-expressing rat kidney KNRK cells (Lipofectamine® method, Gibco, BRL, Gaithersburg, MD, U.S.A.), cultured at 37°C in Dulbecco's Minimal Essential Medium (DMEM, Gibco, BRL, Gaithersburg, MD, U.S.A.) supplemented with 10% v/v fetal calf serum (FCS), under a humidified atmosphere of 5% CO_2 in room air. The cloned human PAR_2 in pcDNA3, was kindly provided by Dr N. Bunnett, University of California at San Francisco, San Francisco, CA, U.S.A. Cell monolayers, grown to about 90% confluency and maintained in the presence of 600 μg ml^{-1} geneticin in 80 cm^2 T-flasks, were used for measurements of trypsin and PAR_2AP-induced calcium signals, essentially as described previously (Hollenberg *et al*., 1997). Cells that had been subcultured without the use of trypsin (split with calcium-free saline containing 1 mM EDTA) were rinsed and disaggregated using isotonic calcium-free phosphate-buffered saline. After harvesting by centrifugation, cells were resuspended in 1 ml of DMEM-10% FCS for loading cells with the intracellular calcium indicator, fluo-3 (Molecular Probes Inc., Eugene, OR, U.S.A.), at a final concentration of 25 μg ml^{-1} (22 μM) of fluo-3 AM ester. Cell uptake was allowed to proceed for 20 to 25 min at room temperature in the presence of 0.25 mM sulfinpyrazone, at which point the dye-loaded cells were washed free of excess fluo-3 AM by centrifugation and resuspended in a buffer, pH 7.4, having a composition (mM): NaCl 150, KCl 3, $CaCl_2$ 1.5, HEPES 20, glucose 10 and sulfinpyrazone 0.25 (stock cell suspension, approx. 10^6 cells ml^{-1}). Fluorescence measurements, monitoring elevations of intracellular calcium (E_{530}) were done at 24°C using an excitation wavelength of 480 nm and emission recorded at 530 nm. Cells (approximately 2 ml of about 3×10^5 cells ml^{-1}) were maintained in suspension in a thermostatted plastic 4 ml cuvette (magnetic flea-bar) maintained at 24°C; peptide or trypsin stock solutions were added directly to the suspension to monitor agonist-induced changes in fluorescence. To construct concentration-effect curves, the peak fluorescence responses to a test concentration of each agonist were expressed as a percentage (% A23187) of the fluorescence peak height measured in replicate cell suspensions upon the addition of 2 μM A23187 (Sigma Chemical Corp., St. Louis, MO, U.S.A.). Data points represent the mean ± s.e.mean (bars) for 3 to 6 measurements done in triplicate, using two or more separately grown crops of human PAR_2-KNRK cells.

Peptides and other reagents

All peptides, prepared by solid phase synthesis followed by HPLC purification, were obtained either from the Peptide Synthesis Facility of the University of Calgary, Faculty of Medicine, Calgary, AB Canada (Director, Dr D. McMaster) or through the courtesy of Dr L. Leblond *via* the peptide synthesis facility at BioChem Therapeutic (Laval, PQ, Canada). The composition of the HPLC-purified ($\geqslant$98% purity) peptides was confirmed by mass spectral analysis. Stock solutions of peptides, prepared in 25 mM HEPES buffer, pH 7.4, were analysed by quantitative amino acid analysis to verify peptide concentration and purity. Porcine trypsin (14,900 u mg^{-1}, Cat. No. T7418), thrombin (3000 NIH u mg^{-1}, from human plasma, free of other clotting factors, CAT. No. T6759) acetylcholine, indomethacin, prazosin, histamine, atropine, chlorpheniramine, phenylephrine, L-NAME, and BQ123 (cyclo-[D-Asp-Pro-D-Val-L-Leu-D-Trp]) and tetrodotoxin were from Sigma (St. Louis MO, U.S.A.). MK886 the leukotriene synthesis inhibitor (3-[1-(4-chlorobenzyl) - 3-t-butyl - thio-t-isopropyl-indol-2-yl]-2,2-dimethylpropanoic acid) was from the Merck-Frost Canada (Pointe Claire-Dorval, PQ, Canada), the nonspecific P450 (epoxygenase) inhibitor, SKF-525A (β-diethylaminoethyl-diphenyl-propylacetate HCl), was from Sigma (St. Louis, MO, U.S.A.) and DuP753 (Losartan®) was from Merck U.S.A. (Rahway, NJ, U.S.A.). Phenoxybenzamine was from Research Biochemicals International (Natick, MA, U.S.A.). A maximum specific activity of 20,000 u mg^{-1} was used to calculate the approximate molar concentration of trypsin in the organ bath.

Results

Actions of SLIGRL-NH$_2$, trypsin, TFLLR-NH$_2$ and thrombin in endothelium-intact versus endothelium-demanded HUV tissue

In our initial experiments, we evaluated that contractile actions of SL-NH$_2$ (5–50 μM), trypsin (100 u ml^{-1}, 200 nM), TF-NH$_2$ (2.5–50 μM) and thrombin (2–10 u ml^{-1}, 20–100 nM) in either intact (Figures 2A and 3A) or endothelium-denuded (Figures 2B and 3B) HUV ring preparations. The reverse peptide, LSIGRL-NH$_2$ that cannot activate PAR$_2$ was also tested. Uniformly (over 20 different preparations initially examined) we found that endothelium-intact preparations (Figures 2A and 3A) contracted in response to the PAR$_2$AP, SL-NH$_2$ (e.g. 5 μM) but not either to much higher concentrations of the PAR$_1$-selective agonist peptide, TF-NH$_2$ (e.g. 50 μM (Figure 2A) or to the PAR$_2$-activating enzyme, trypsin (Figure 3A); the nonselective PAR$_1$/PAR$_2$ agonist, SFLLR-NH$_2$ (50 μM) was similarly inactive (not shown). In contrast in endothelium-denuded preparations, both TF-NH$_2$ at relatively low concentrations (e.g. 2.5 μM (Figures 2B and 3B) and thrombin (Figure 3B) caused a prompt contractile response. Further, in the endothelium-intact HUV preparation, the continual presence in the organ bath of a high concentration of TF-NH$_2$ (50 μM) did not block the contractile action of the 10 fold lower concentration of SL-NH$_2$ in the endothelium-intact preparation (Figure 2A). In a similar vein, the presence of a high concentration of SL-NH$_2$ (e.g. 50 μM) in the organ bath did not, in an endothelium-denuded preparation, block the contractile action of a more than 10 fold lower concentration of TF-NH$_2$ (e.g. 2.5 μM, Figure 2B). The partial reverse-sequence peptide, LSIGRL-NH$_2$ caused no effect in either the intact of endothelium-denuded preparation. Thus, the responses to the PAR$_2$AP, SL-NH$_2$ (endothelium-intact) and the PAR$_1$AP, TF-NH$_2$ (endothelium-denuded) appeared to be mutually exclusive, depending on whether or not the preparation had been stripped of its endothelium. It was of

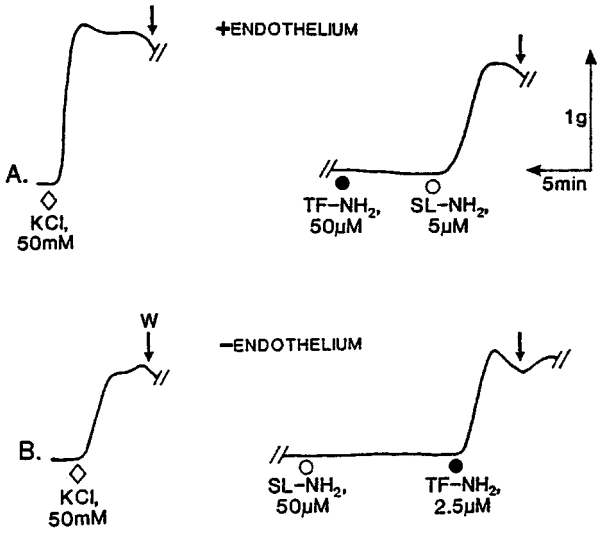

Figure 2 Contractile responses of endothelium-intact (A) and endothelium-denuded (B) preparations to PAR-APs. The contractile responses of either endothelium-intact (A) or endothelium-denuded (B) preparations to the PAR$_1$-selective agonist. TFLLR-NH$_2$ and the PAR$_2$-selective agonist, SLIGRL-NH$_2$ were monitored in preparations that were either intact (A) or were denuded of endothelium (B). Each tissue was first tested for its contractile response to 50 mM KCl, followed by a tissue wash (W, arrows). PAR agonists were added sequentially to the organ bath without an intervening tissue wash. The scale for time (min) and tension (g) is shown on the right. The tracings are representative of over 15 comparable experiments with independently prepared HUV rings from different donors.

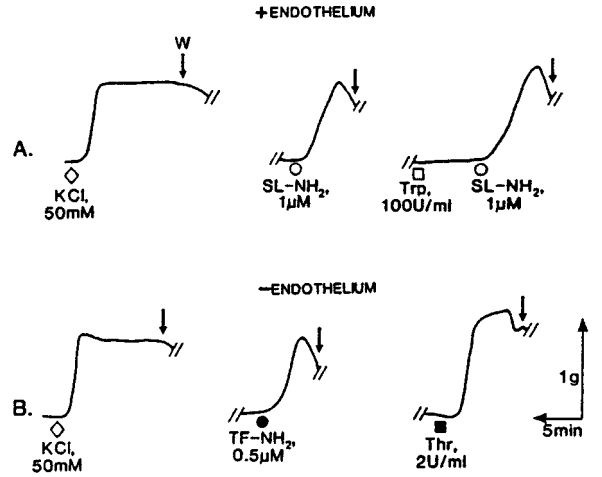

Figure 3 Comparative contractile actions of PAR-APs with trypsin (A) and thrombin (B) in either endothelium intact (A) or endothelium-denuded (B) HUV ring preparations. Either intact (A) or endothelium-denuded (B) HUV ring preparations were first assessed for contractile responsiveness by the addition of KCl to the organ bath followed by a tissue wash. The endothelium-intact preparation (A) was then challenged both with the PAR$_2$AP, SLIGRL-NH$_2$ and after a tissue wash, with trypsin followed by the repeat addition of SL-NH$_2$. The endothelium-denuded preparation (B) was challenged both with the PAR$_1$-selective peptide TFLLR-NH$_2$ and after a tissue wash, with thrombin. The scale for time (min) and tension (g) is shown on the right. The tracings are representative of more than seven independently conducted experiments with different HUV preparations coming from different donor tissues.

note that a concentration of trypsin (100 u ml^{-1}; 200 nM), far above that expected to activate PAR_2 (e.g. 20 u ml^{-1} Nystedt *et al*., 1994; 1995a,b), was not able to cause a contractile response in an endothelium-intact preparation that responsed well to a comparatively low concentration of SL-NH_2 (1 μM: Figure 3A).

Effects of antagonists on the contractile action of SL-NH_2 in endothelium-intact HUV tissue

To determine if the contractile action of SL-NH_2 in the endothelium-intact HUV preparation might be due to a contractile metabolic product of arachidonic acid (*via* cyclooxygenase, lipoxygenase or epoxygenase), or to the release of endothelin (Magazine *et al*., 1996) we assessed the contractile action of SL-NH_2 in the presence of BQ123 (an ET_A receptor antagonist), indomethacin (cyclooxygenase inhibitor), MK886 (inhibitor of leukotriene synthesis) and SKF-525A (inhibitor of epoxygenase/cytochrome P450 oxygenase). Each of these reagents was assessed in three or more replicate HUV preparations, for which representative tracings are shown in Figure 4. None of the enzyme inhibitors at concentrations known to inhibit enzyme activity (3–10 μM) blocked the contractile action of 2.5 μM SL-NH_2, (Figure 4B–D); and BQ123 (1 μM), at a concentration sufficient to block completely the contractile action of 10 nM endothelin (not shown; and see Figure 7), had no effect on the contractile action of 2.5 μM SL-NH_2 (Figure 4A). Similarly, the following pharmacological antagonists (all at 1 μM) had no effect on the contractile action of SL-NH_2 in the endothelium-intact HUV preparation: phenoxybenzamine, prazosin, DuP753 (Losartan®), tetrodotoxin and chlorpheniramine; the concentrations of antagonists used blocked the contractile actions of the specific agonists in the tissue (not shown). Since 1 μM acetylcholine had no effect on the tension of endothelium-intact HUV preparations (not shown), muscarinic antagonists were not evaluated.

Effects of agonists in causing contractile and relaxant responses in the reporter tissue assay

The 'sandwich assay', employing the endothelium-denuded rat aorta ring as a reporter tissue (Figure 1), was used to detect the release of either contractile (Figure 5) or relaxant (Figure 6) substances from either endothelium-intact or endothelium-denuded HUV tissue. As shown in Figure 5, contractile activity was released from the L-NAME-treated endothelium-intact preparation by SL-NH_2 but not by trypsin (Figure 5A). No such activity was released by SL-NH_2 or trypsin from the endothelium-denuded HUV tissue (Figure 5B).

As shown in Figure 6A–C, histamine, as previously reported (Van de Voorde *et al*., 1987; Chaudhuri *et al*., 1991), was able to cause release of relaxant activity from the endothelium-intact HUV preparation. In the absence of the HUV tissue, 20 μM histamine did not affect the tension of the aorta reporter tissue; and the relaxant response to histamine was not observed when either endothelium-denuded HUV tissue or endothelium-intact HUV tissues were treated with 0.1 mM L-NAME (not shown). As illustrated in Figure 6A, neither thrombin (10 u ml^{-1}, 100 nM) nor the selective PAR_1AP, TFLLR-NH_2 (50 μM) were able to cause the release of relaxant activity from an endothelium-intact HUV preparation in which trypsin (5 u ml^{-1}; 10 nM) was able to do so. Further, the trypsin-induced release of relaxant activity was abolished in the presence of 0.1 mM L-NAME (Figure 6B) as was the comparable action of SLIGRL-NH_2, in preparation that was otherwise refractory to the release of relaxant activity

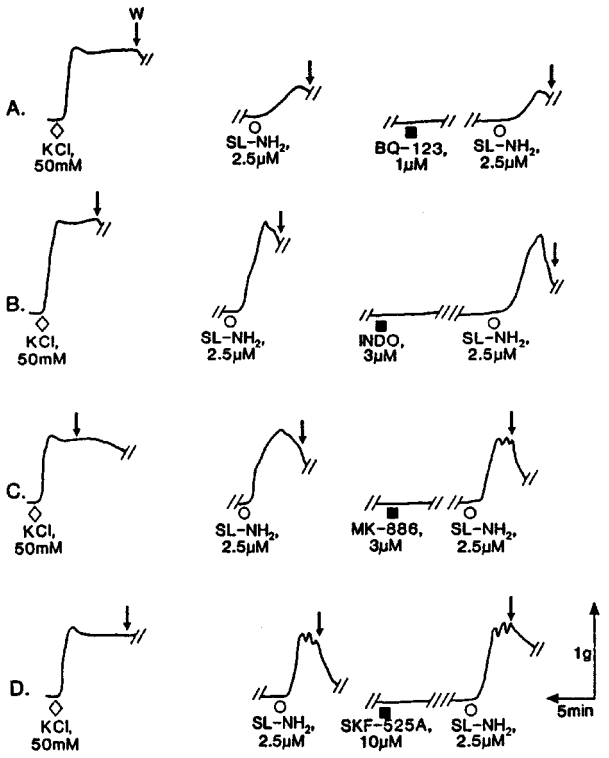

Figure 4 Effects of an endothelin receptor antagonist and inhibitors of cyclooxygenase, leukotriene synthesis and epoxygenase on PAR_2AP-induced contractions in the endothelium-intact HUV preparation. Individual endothelium-intact HUV preparations (A – D) were first tested for their contractile responses by exposure to KCl followed by a tissue wash (W, arrows) and then by the addition of the selective PAR_2AP, SLIGRL-NH_2. Subsequent to a second tissue wash, each preparation was treated for 15 min with the following inhibitors (A) BQ123; (B) indomethacin; (C) MK-886 and (D) SKF-525A. In the continued presence of each inhibitor, the tissues were then challenged again with 2.5 μM SL-NH_2. At the concentration used, BQ123 (1 μM) completely blocked the action of 10 nM endothelin-1 (not shown). The tracings are representative of four or more independently conducted experiments with HUV preparations derived from different donors. The scale for time and tension is shown to the right of (D).

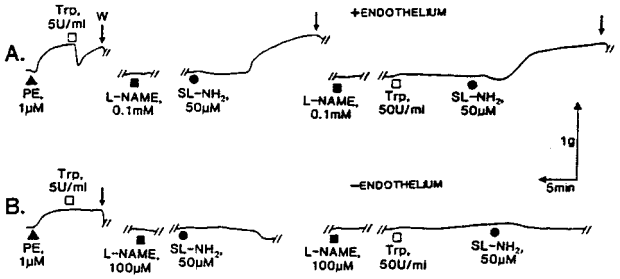

Figure 5 Release of EDCF by SLGRL-NH_2: dependence on an intact HUV endothelium and lack of inhibition by L-NAME. The presence (A) or absence (B) of an intact HUV endothelium was first established by the presence (A) or absence (B) of a relaxation of the preconstricted aorta reporter tissue, upon adding either 20 μM histamine (not shown) or trypsin to the organ bath, followed by a tissue wash. Tissues were then pretreated with 0.1 mM L-NAME and the contractile response (EDCF release) of the aorta reporter tissue was then evaluated, upon addition to the organ bath of SLGRL-NH_2 (middle and right-hand A and B) and trypsin (right-hand A and B). (A) Sandwich preparation with an intact HUV endothelium. (B) Sandwich preparation with an endothelium-denuded HUV tissue. The scale for time (min) and tension (g) is shown between the two tracings. (A and B) Each tracing showing the response of an individual sandwich preparation is representative of seven or more independently conducted experiments with different HUV preparations.

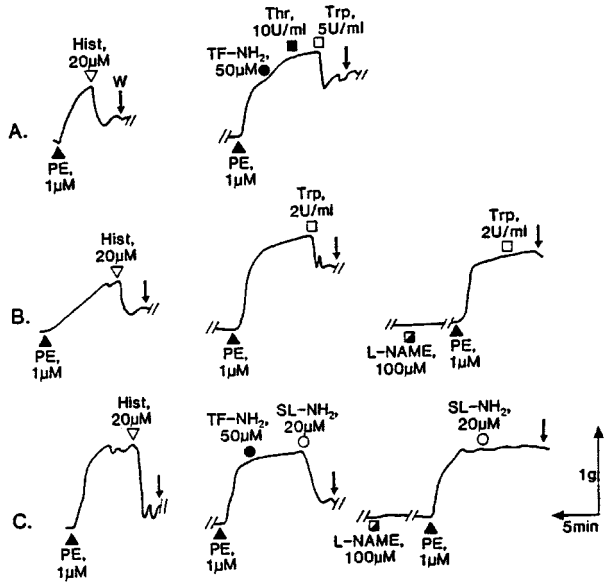

Figure 6 Relaxation of the preconstricted aorta reporter tissue in the 'sandwich' assay upon exposure of the donor HUV tissue to histamine, trypsin and SLIGRL-NH_2, but not to thrombin and the selective PAR_1AP, TFLLR-NH_2: inhibitory action of L-NAME. Individual endothelium-intact HUV preparations were first tested with histamine for their ability to relax a phenylphrine-preconstricted aorta reporter tissue (left-hand A–C). After a tissue wash, each HUV preparation was then tested for its ability to relax the preconstricted reporter tissue in response to thrombin, trypsin, TFLLR-NH_2 (A and C) and SLIGRL-NH_2 (C). The relaxant effects of trypsin (B) and SL-NH_2 (C) were also monitored after a 20 min pretreatment of tissues with 100 μM L-NAME (B for trypsin; C for SL-NH_2). The tracings representing an experiment with individual HUV preparations (A–C) are each representative of five or more independently conducted experiments with different HUV donor tissues.

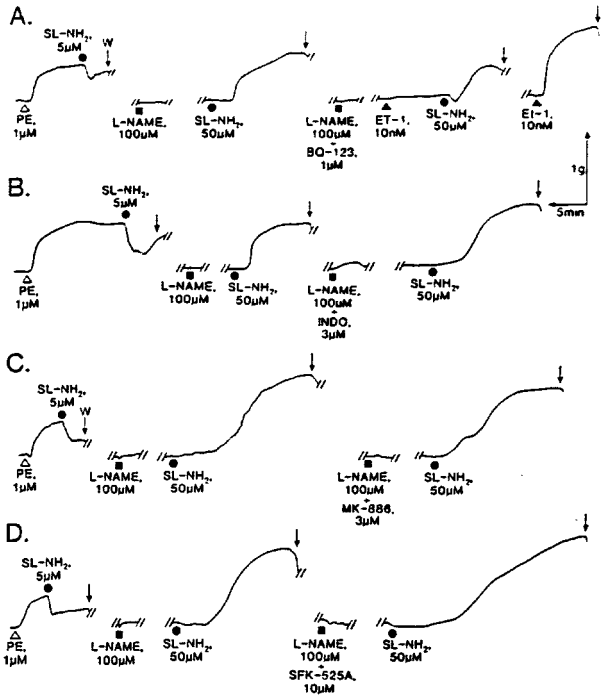

Figure 7 Effects of an endothelin receptor antagonist and inhibitors of cyclooxygenase, leukotriene synthesis and epoxygenase on PAR_2-induced aorta reporter tissue contractions in the HUV 'sandwich' assay. As outlined for the direct HUV contractions assay in the legend to Figure 4, the following inhibitors were evaluated for their ability to block SL-NH_2-mediated contraction of the reporter aorta tissue in the HUV 'sandwich' assay: (A) BQ123; (B) indomethacin; (C) MK-886 and (D) SKF-525A. Sandwich assays employing endothelium-intact HUV tissue were first assessed (left-hand A–D) for the ability of added SL-NH_2 to cause a relaxation of the phenylephrine-preconstricted aorta reporter tissue, thereby confirming the presence of a functional endothelium, followed by a tissue wash. Each preparation was then treated for 20 min with 0.1 mM L-NAME, and a contraction of the aorta reporter tissue upon adding SL-NH_2 to the organ bath was monitored (middle A–D). Thereafter each preparation was treated for 20 min with the indicated inhibitor, and the contractile responses of the inhibitor-treated preparations were again monitored. The ability of 1 μM BQ123 to block contractions caused by the addition of endothelin-1, but not SL-NH_2 was evaluated in (A). The return of the ET-1-mediated contraction upon washing out BQ123 is shown on the right in (A). (A–D) Each showing the response of an individual HUV sandwich preparation are representative of three or more separately conducted experiments with different HUV sandwich preparations. The scale for time (min) and tension (g) is shown on the right between (A) and (B).

by 50 μM TFLLR-NH_2 (TF-NH_2 Figure 6C). Moreover, the presence of an excess of TF-NH_2 in the organ bath did not affect the ability of SLIGRL-NH_2 to cause the release of relaxant activity from the endothelium-intact HUV preparation (Figure 6C). The release of relaxant activity by trypsin (e.g. Figure 6A and B and Figure 5A) was dependent on the presence of an intact endothelium. No release of relaxant activity in response to trypsin (Figure 5B) or SLIGRL-NH_2 (not shown) was observed when the HUV preparation had been denuded of endothelium. Such endothelium-denuded preparations also failed to release relaxant activity in response to 20 μM histamine (not shown).

Effects of inhibitors on the release of contractile activity from endothelium-intact HUV tissue

Using the 'sandwich' assay, we assessed the same matrix of enzyme inhibitors and receptor antagonists that we had previously evaluated in the HUV contraction assay (above, Figure 4) for their ability to affect the release/action of contractile activity from endothelium-intact HUV tissue (Figure 7). Significantly, BQ123 failed to block the contractile action of the factor released by SL-NH_2 from the HUV tissue, whereas the contractile action of 10 nM endothelin-1 was completely abrogated (Figure 7A). Similarly, as for the HUV contractile assay, indomethacin, MK886 and SKF-525A had no effect on the release or action of the HUV-derived contractile factor (Figure 7B–D). Other receptor antagonists (prazosin and Dup753 [Losartan®]) were similarly without effect (not shown). Since acetylcholine, histamine and 5-hydroxytryptamine did not cause a contractile effect in the aorta reporter tissue, antagonists of these agents were not evaluated in the 'sandwich assay' (not shown).

Contractile concentration-effect curves for PAR_2APs in endothelium-intact HUV tissue

Concentration-effect curves were obtained for the contractile actions of the PAR_2APs, SLIGRL-NH_2, SLIGKV-NH_2, and trans-cinnamoyl-LIGRLO-NH_2 in the endothelium-intact HUV preparation (Figure 8). The order of potencies of the PAR_2APs were: SL-NH_2 > > KV-NH_2 $\geqslant$ tc-NH_2. Significantly, neither trypsin (0.2 μM; 100 u ml^{-1}) nor TF-NH_2 (up to 50 μM) were active in the endothelium-intact HUV contraction assay (Figure 8). The relative activities of the PAR_2APs in the HUV contraction assay are summarized in Table 1.

Concentration-effect curves for the release of relaxant activity from HUV tissue

Although we were able to assess the structure-activity relationships (SARs) for the direct contractile action of PAR_2APs in the HUV tissue, it did not prove possible to evaluate directly the SARs for the relaxant activity of the peptides. The reason for this situation was that, as we had observed previously for PAR_1APs (Tay-Uyboco *et al*., 1995), neither PAR_1APs nor PAR_2APs caused a relaxation in an endothelium-intact HUV preparation that had been precontracted with phenylephrine (not shown). We determined that even the addition of NO-donors (10 μM sodium nitroprusside) or NO itself (up to 1 μM) failed to cause relaxation in a preconstricted HUV preparation (not shown). Thus, we turned to the sandwich assay as a means of evaluating the structure-activity relationships for the ability of trypsin, SLIGRL-NH_2, SLIGKV-NH_2, trans-cinnamoyl-LIGRLO-NH_2 and TFLLR-NH_2 to stimulate the release of EDRF (presumably, NO) from the endothelium-intact HUV preparation (Figures 6 and 9). From the concentration-effect curves shown in Figure 9, the relative potencies of trypsin and the PAR_2APs for stimulating NO release were: trypsin > > SL-NH_2 > > tc-NH_2 > KV-NH_2; both thrombin (up to 1 μM) and TF-NH_2 (up to 50 μM) were inactive in the assay, as also illustrated above in Figure 6. The relative activities of trypsin and the PAR_2APs in the 'sandwich' relaxation assay are summarized and expressed quantitatively in Table 1.

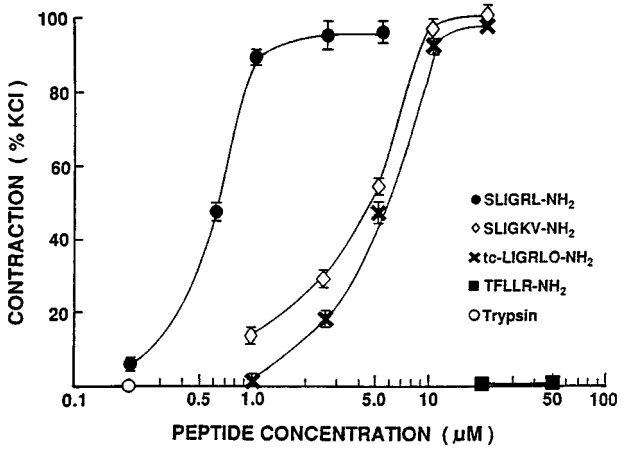

Figure 8 Concentration-response curves for the contractile actions of PAR_2APs in the endothelium-intact HUV preparation. The contractile responses of endothelium-intact HUV preparations were measured for different concentrations of the PAR_2APs, SLIGRL-NH_2, SLIGKV-NH_2 and trans-cinnamoyl LIGRLO-NH_2 and were expressed as a percentage (% KCl) of the contractile response of each preparation to 50 mM KCl. The responsiveness of comparable preparations to either trypsin or the selective PAR_1AP, TFLLR-NH_2 was also evaluated. Data points represent the mean ± s.e.mean (bars) for six or more measurements made with HUV preparations derived from more than four different donor tissues.

Table 1 Relative activities for PAR_2APs and trypsin in the contraction, relaxation and calcium signalling assays

	Relative activity ratio (R_{EC})[a] assay		
Agonist	*Contraction*	*Relaxation*	*Calcium*
SL-NH_2	1	1	1
KV-NH_2	8.1 ± 0.1	4.4 ± 0.2	2.7 ± 0.1
tc-NH_2	8.8 ± 0.2	3.2 ± 0.2	1.0 ± 0.0
Trypsin ($\times 10^3$)	N.A.[b]	5.8 ± 0.1	2.4 ± 0.1

[a]Activity ratios (R_{EC}) for each agonist, relative to the concentration of SL-NH_2 (R_{EC} = 1) causing an equivalent biological response, were calculated along lines previously described (Tay-Uyboco *et al*., 1995) from the concentration-response curves shown in Figures 8, 9 and 10, according to the formula; R_{EC} = (concentration of agonist for a given response in the linear portion of its concentration-effect curve) ÷ (concentration of SL-NH_2 required to cause the equivalent biological response). A value greater than 1.0 designates an agonist with a potency lower than that of SL-NH_2. Values for trypsin were multiplied by 10^3. Each value represents an average (± s.e.mean) obtained for concentration ratios measured at five levels of response along the concentration-response curves for each agonist. [b]N.A. = not active.

Relative activities of trypsin and the PAR_2APs for activating the cloned human PAR_2 expressed in KNRK cells

The ability of trypsin and the PAR_2AP, SLIGKV-NH_2 to activate cloned human PAR_2 expressed in KNRK cells had been observed previously (Böhm *et al*., 1996); but no structure-activity data for the PAR_2APs that we had used in the HUV contraction and relaxation assay were available. Therefore, we expressed human PAR_2 in the KNRK cells, and we used a PAR_2AP-stimulated calcium signalling assay (Hollenberg *et al*., 1997) to assess the relative potencies of trypsin and the three PAR_2APs for activating the receptor. As shown in Figure 10 and summarized quantitatively in Table 1, the relative potencies in the calcium signalling assay (trypsin > > SL-NH_2 = tc-NH_2 > KV-NH_2) differed both from the potency order observed in the 'sandwich' relaxation assay and from the order of potencies observed in the HUV contraction assay.

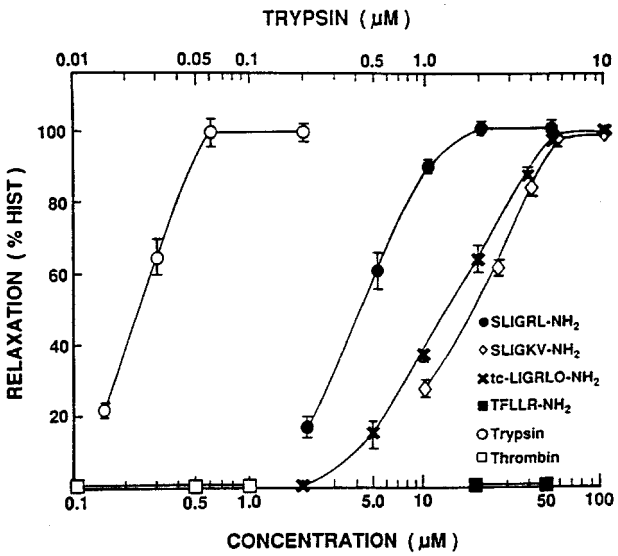

Figure 9 Concentration-effect curves for the relaxant effects of PAR_2APs and trypsin in the HUV 'sandwich' assay. The relaxant effects of different concentrations of SLIGRL-NH_2, SLIGKV-NH_2 trans-cinnamoyl-LIGRLO-NH_2 and trypsin were measured in the HUV 'sandwich' relaxation assay, using endothelium-intact HUV donor tissue. The relaxant response was expressed as a percentage (% HIST) of the relaxation caused by 20 μM histamine in the phenylephrine-preconstricted aorta reporter tissue (see Figure 6). The effects of adding TFLLR-NH_2 thrombin and trypsin to the organ bath were also measured. Each data point represents the mean ± s.e.mean (bars) for measurements with five or more individual HUV sandwich preparations derived from three or more HUV tissues.

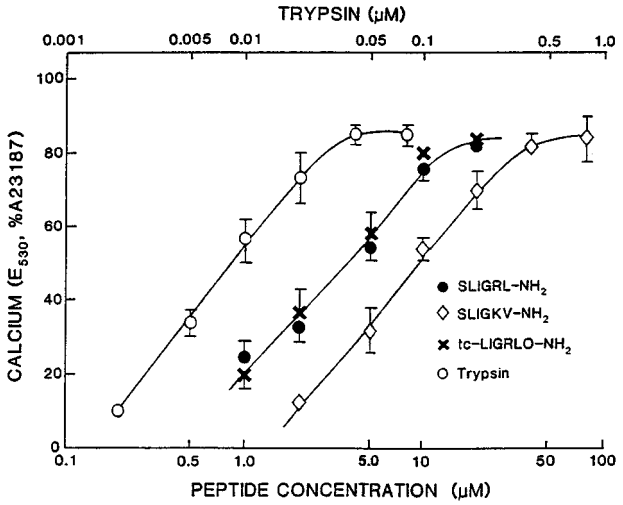

Figure 10 Concentration-effect curves for PAR_2APs and trypsin in the KNRK-PAR_2 cell calcium signalling assay. The effects on fluo-3 fluorescence of increasing concentration of trypsin and the PAR_2APs, SLIGRL-NH_2 SLIGKV-NH_2 (◇) and trans-cinnamoyl-LIGRLO-NH_2 were measured as a percentage (% A23187) of the fluorescence signal (E_{530}) caused by the addition of 2 μM of the ionophore, A23187 to replicate fluo-3-loaded cell suspensions. Values represent the means ± s.e.mean for 2 – 3 independently conducted experiments with replicate cell suspensions derived from independently grown crops of KNRK-PAR_2 cells. Error bars smaller than the symbols are not shown.

Discussion

Release of a novel EDCF via *a receptor distinct from PAR_2*

The principal finding of our study was that the PAR_2APs (but not trypsin) were able to cause the release of a diffusable endothelium-derived contracting factor (EDCF) from endothelium-intact HUV ring preparations. In contrast, both trypsin and PAR_2APs stimulated the release of an L-NAME-sensitive relaxing factor (presumably NO) from the same tissue. Our new data considerably extend our previous finding of both a contractile and relaxant action of PAR_2APs in endothelium-intact rat arterial rings (Roy *et al*., 1998). As a major advantage over our previous work with rat tissue, the 'sandwich' assay that we were able to use with the HUV tissue enabled us to demonstrate that the HUV contractile response was due to the release of a diffusable EDCF rather than as a result of electrical coupling between the endothelial cells and the underlying HUV smooth muscle elements. Our data indicated that this EDCF was distinct from noradrenalin, angiotensin and endothelin; and from other contractile agonists that might have been formed by the metabolism of arachidonic acid. Given that the HUV tissue released a sufficient amount of the EDCF into the organ bath to affect the rat aorta reporter tissue, we are optimistic that further work will be able to characterize the nature of the substance(s) released into tissue bath medium. The release of EDRF by both trypsin and PAR_2APs is in agreement with previous work (Chaudhuri *et al*., 1991) demonstrating EDRF release from HUV tissue and is in keeping with the landmark work of Furchgott & Zawadzki (1980); this EDRF is now known to be NO.

In accord with our previous work with rat vascular tissue (Roy *et al*., 1998), trypsin, at concentrations that were more than sufficient to activate PAR_2 (Figure 10), was not able to cause a contractile response in the endothelium-intact HUV tissue (Figure 3) and was not able to cause the release of EDCF (Figure 5). It was also of significance that the non-selective PAR-AP, SFLLR-NH_2 which is fully capable of activating both PAR_1 and PAR_2 (Hollenberg *et al*., 1997; Blackhart *et al*., 1996), was unable to cause a contractile response in endothelium-intact HUV tissue (data not shown and see Figure 2 of Tay-Uyboco *et al*., 1995). Our new data with the endothelium-intact HUV tissue did, nonetheless, differ from our previous observations with the rat vascular preparation, in that the PAR_2AP, tc-NH_2 was active in causing a contraction in the HUV preparation, whereas this peptide analogue, lacking a free NH_2 group at the amino-terminus, was inactive as a contractile agonist in the endothelium-intact rat arterial ring preparation (Roy *et al*., 1998). In earlier work, we had demonstrated that peptide metabolism by HUV tissue in the organ bath cannot account for distinct peptide SARs in this preparation (Tay-Uyboco *et al*., 1995). Notwithstanding: (1) the very distinct SAR relationships for the three PAR_2APs in the HUV contraction assay, compared with the relaxation assay and the transfected PAR_2 calcium signalling assay (summarized in Table 1). (2) the lack of activity of trypsin in the HUV contraction assay (Figures 3A and 8) and (3) the lack of activity of the non-selective PAR-AP, SFLLR-NH_2 in the HUV contraction assay (data not shown and Tay-Uyboco *et al*., 1995) all point to an endothelial receptor, distinct from PAR_2, that is responsible for the release of a novel diffusable EDCF. It is unlikely that this receptor is PAR_4, since thrombin was unable to cause a contractile response in endothelium-intact HUV tissue (this study and Tay-Uyboco *et al*., 1995). The contractile responsiveness of the HUV receptor to tc-NH_2 in contrast with the receptor present in rat endothelial tissue (Roy *et al*., 1998), suggests that the rat and human endothelial receptors may possibly represent related but distinct 'subtypes'. Whether or not the PAR_2AP-responsive trypsin-resistant receptor in the umbilical vein endothelium that is responsible for the release of EDCF can be activated by other proteinases remains to be determined. In contrast with the receptor responsible for EDCF release, the HUV endothelial receptor responsible for EDRF (NO) release had all the hallmarks of PAR_2 (activated both by low concentrations of trypsin and by PAR_2APs) (see however, Discussion).

Release of NO via *PAR_2 and not PAR_1 from HUV tissue*

Our new data with the 'sandwich'-based relaxation assay and with the contractile assay using endothelium-denuded HUV tissue have clarified several issues that arose from our previous work (Tay-Uyboco *et al*., 1995) with the human umbilical vein. First, it is now clear that our inability to observe a relaxant response to PAR_1 activation in HUV tissue (Figure 2, Tay-Uyboco *et al*., 1995) was due to two factors: (1) our new data show (Figure 6) that stimulation of the endothelium-intact HUV preparation either by thrombin or by the selective PAR_1AP, TF-NH_2, failed to cause the release of EDRF (very likely NO) from the tissue and (2) our work has now found that the HUV tissue *per se* is particularly insensitive to the relaxant actions of either NO donors (e.g. nitroprusside) or NO itself. As opposed to the release of EDRF from the same preparation caused by (presumed) PAR_2 activation (trypsin and SL-NH_2 in the 'sandwich' assay, Figure 6), the absence of EDRF release in response to either thrombin or TF-NH_2 suggests that functional thrombin receptors are probably absent from the HUV endothelium *in vitro*, where only functional PAR_2 can be detected pharmacologically. In contrast, both PAR_1 and PAR_2 can be observed to be

functional in cultured endothelial cells derived from the human umbilical vein (Mirza *et al*., 1996). Our work with the intact HUV tissue should thus alert those who work with cultured endothelial cells to the considerable differences in PAR dynamics that may be present in cultured endothelial cell systems, compared with intact tissues *in vivo*.

Distinct structure-activity relationships for PAR_2APs in the HUV tissue and in KNRK-PAR_2 cells

An issue that we cannot yet resolve relates to the different structure-activity relationships we have observed from trypsin and the PAR_2APs in the 'sandwich' relaxation assay, compared with the cloned human KNRK-PAR_2 calcium signalling assay (Table 1). According to the ability of trypsin and the three PAR_2APs (but not the PAR_1-selective peptide, TF-NH_2) to stimulate EDRF release from the endothelium-intact tissue, the HUV endothelial receptor undoubtedly belongs to the family of proteinase-activated receptors. But, is it the same PAR_2 that has been cloned and evaluated in tissue and cellular expression assays by others (Nystedt *et al*., 1995b; Böhm *et al*., 1996) and by us (Saifeddine *et al*., 1996; Vergnolle *et al*., 1998)? The distinct SARs for the relaxation and calcium signalling assays shown in Figures 9 and 10 and summarized in Table 1, where there is clearly a discrepancy for the relative activity of tc-NH_2, would suggest that the answer to this question may be no; and that a distinct PAR_2 subtype may be present in the HUV endothelium. Unfortunately, effective and selective PAR_2 antagonists are not yet available to deal directly with the above question. It is well recognized that the relative potencies of a series of agonist can in theory differ, depending on the tissue background against which an individual receptor mRNA may be expressed (e.g. McIntyre *et al*., 1993). Thus, differences in the relative potencies of a series of agonists (Ahlquist, 1948) can only be taken as preliminary evidence for the existence of distinct receptor subtypes. Further work will be required to evaluate not only the distinct trypsin-resistant, PAR_2AP-sensitive endothelial receptor responsible for the release of EDCF from the HUV tissue, but also the trypsin-activated endothelial receptor in the HUV responsible for EDRF (NO) release. Moreover, it will be a challenge to characterize what we believe to be a novel EDCF that can be released from the HUV tissue by PAR_2APs, but not by PAR_1APs or trypsin.

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References

AHLQUIST, R.P. (1948). A study of the adrenotropic receptors. *Am. J. Physiol.,* **153,** 586 – 600.

AL-ANI, B., SAIFEDDINE, M. & HOLLENBERG, M.D. (1995). Detection of functional receptors for the proteinase-activated receptor-2-activating polypeptide, SLIGRL-NH_2 in rat vascular and gastric smooth muscle. *Can. J. Physiol. Pharmacol.,* **73,** 1203 – 1207.

BLACKHART, B.C., EMILSSON, K., NGUYEN, D., TENG, W., MARTELLI, A.J., NYSTEDT, S., SUNDELIN, J. & SCARBOROUGH, R.M. (1996). Ligand cross-reactivity within the protease-activated receptor family. *J. Biol. Chem.,* **271,** 16466 – 16471.

BÖHM, S.K., KONG, W., BRÖMME, D., SMEEKENS, S.P., ANDERSON, D.C., CONNOLLY, A., KAHN, M., NELKEN, N.A., COUGHLIN, S.R., PAYAN, D.G. & BUNNETT, N.W. (1996). Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem. J.,* **314,** 1009 – 1016.

BUTTERY, L.D.K., MCCARTHY, A., SPRINGALL, D.R., SULLIVAN, M.H.F., ELDER, M.G., MICHEL, T. & POLAK, J.M. (1994). Endothelial nitric oxide synthase in the human placenta: regional distribution and proposed regulatory role at the feto-material interface. *Placenta,* **15,** 257 – 265.

CHAUDHURI, G., BUGA, G.M., GOLD, M.E., WOOD, K.S. & IGNARRO, L.J. (1991). Characterization and actions of human umbilical endothelium derived relaxing factor. *Br. J. Pharmacol.,* **102,** 331 – 336.

CORVERA, C.U., DÉRY, O., MCCONALOGUE, K., BOHM, S.K., KHITIN, L.M., CAUGHEY, G.H., PAYAN, D.G., BUNNETT, N.W. (1997). Mast cell tryptase regulates rat colonic myocytes through proteinase-activated receptor 2. *J. Clin. Invest.,* **100,** 1383 – 1393.

COUGHLIN, S.R., VU, T.-K., HUNG, D.T. & WHEATON, V.I. (1992). Characterization of a functional thrombin receptor. *J. Clin. Invest.,* **89,** 351 – 355.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature,* **288,** 373 – 376.

HOLLENBERG, M.D., SAIFEDDINE, M. & AL-ANI, B. (1996). Proteinase-activated receptor-2 in rat aorta: structural requirements for agonist activity of receptor-activating peptides. *Mol. Pharmacol.,* **49,** 229 – 233.

HOLLENBERG, M.D., SAIFEDDINE, M., AL-ANI, B & KAWABATA, A. (1997). Proteinase-activated receptors: structural requirements for activity, receptor cross-reactivity and receptor selectivity of receptor-activating peptides. *Can. J. Phys. Pharmacol.,* **75,** 832 – 841.

ISHIHARA, H., CONNOLLY, A.J., ZENG, D., KAHN, M.L., ZHENG, Y.W., TIMMONS, C., TRAM, T. & COUGHLIN, S.R. (1997). Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature,* **386,** 502 – 506.

KAHN, M.K., ZHENG, Y.-W., HUANG, W., BIGORNIA, V., ZENG, D., MOFF, S., FARESE JR, R.V., TAM, C. & COUGHLIN, S.R. (1998). A two thrombin receptor system for platelet activation. *Nature,* in press.

LANIYONU, A.A. & HOLLENBERG, M.D. (1995). Vascular actions of thrombin receptor-derived polypeptides: structure-activity profiles for contractile and relaxant effects in rat aorta. *Br. J. Pharmacol.,* **114,** 1680 – 1686.

MAGAZINE, H.I., KING, J.M. & SRIVASTAVA, K.D. (1996). Protease activated receptors modulate aortic vascular tone. *Intl. J. Cardiol.,* **53** (suppl), S75 – S80.

MCINTYRE, P., PHILLIPS, E., SKIDMORE, E., BROWN, M. & WEBB, M. (1993). Cloned murine bradykinin receptor exhibits a mixed B_1 and B_2 pharmacological selectivity. *Mol. Pharmacol.,* **44,** 346 – 355.

MIRZA, H., SCHMIDT, V.A., DERIAN, C.K., JESTY, J. & BAHOU, W.F. (1997). Mitogenic responses mediated through the proteinase-activated receptor-2 are induced by expressed forms of mast cell α- or β-tryptases. *Blood,* **90,** 3914 – 3922.

MIRZA, H., YATSULA, V. & BAHOU, W.F. (1996). The proteinase activated receptor-2 (PAR-2) mediates mitogenic responses in human vascular endothelial cells. Molecular characterization and evidence for functional coupling to the thrombin receptor. *J. Clin. Invest.,* **97,** 1705 – 1714.

MOLINO, M., BARNATHAN, E.S., NUMEROF, R., CLARK, J., DREYER, M., CUMASHI, A., HOXIE, J.A., SCHECHTER, N., WOOLKALIS, M. & BRASS, L.F. (1997). Interactions of mast cell tryptase with thrombin receptors and PAR-2. *J. Biol. Chem.,* **272,** 4043 – 4049.

NYSTEDT, S., EMILSSON, K., WAHLESTEDT, C. & SUNDELIN, J. (1994). Molecular cloning of a potential proteinase activated receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 9208 – 9212.

NYSTEDT, S., EMILSSON, K., LARSSON, A.K., STRÖMBECK, B. & SUNDELIN, J. (1995a). Molecular cloning and functional expression of the gene encoding the human proteinase-activated receptor 2. *Eur. J. Biochem.,* **232,** 84 – 89.

NYSTEDT, S., LARSSON, A.K., ABERG, H. & SUNDELIN, J. (1995b). The mouse proteinase-activated receptor-2 cDNA and gene. *J. Biol. Chem.,* **270,** 5950 – 5955.

RASMUSSEN, U.G., VOURET-CRAVIART, V., JALLAT, S., SCHLESINGER, Y., PAGES, G., PAVIRANI, A., LECOCQ, J.P., POUYSSEGUR, J. & VAN OBBERGHEN-SCHILLING, E. (1991). cDNA cloning and expression of a hamster α-thrombin receptor coupled to Ca^{2+} mobilization. *FEBS Lett.,* **288,** 123 – 128.

ROY, S.S., SAIFEDDINE, M., LOUTZENHISER, R., TRIGGLE, C.R. & HOLLENBERG, M.D. (1998). Dual endothelium-dependent vascular activities of proteinase-activated receptor-2-activating peptides: evidence for receptor heterogeneity. *Br. J. Pharmacol.,* **123,** 1434 – 1440.

SAIFEDDINE, M., AL-ANI, B., CHENG, C.-H., WANG, L. & HOLLENBERG, M.D. (1996). Rat proteinase-activated receptor-2 (PAR-2): cDNA sequence and activity of receptor-derived peptides in gastric and vascular tissue. *Br. J. Pharmacol.,* **118,** 521 – 530.

TAY-UYBOCO, J., POON, M.-C., AHMAD, S. & HOLLENBERG, M.D. (1995). Contractile actions of thrombin receptor-derived polypeptides in human umbilical and placental vasculature: evidence for distinct receptor systems. *Br. J. Pharmacol.,* **115,** 569 – 578.

VAN DE VOORDE, J., VANDERSTICHELE, H. & LEUSEN, I. (1987). Release of endothelium-derived relaxing factor from human umbilical vessels. *Circ. Res.,* **60,** 517 – 522.

VERGNOLLE, N., MACNAUGHTON, W.K., AL-ANI, B., SAIFEDDINE, M., WALLACE, J.L. & HOLLENBERG, M.D. (1998). Proteinase-activated receptor-2-activating peptides: identification of a receptor distinct from PAR_2 that regulates intestinal transport. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 7766 – 7771.

VU, T.K.H., HUNG, D.T., WHEATON, V.I. & COUGHLIN, S.R. (1991). Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell,* **64,** 1057 – 1068.

XU, W.-F., ANDERSEN, H., WHITMORE, T.E., PRESNESS, S.R., YEE, D.P., CHING, A., GILBERT, T., DAVIE, E.W. & FOSTER, D. (1998). Cloning and characterization of human protease-activated receptor 4. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 6642 – 6646.

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