Modified Proteinase-Activated Receptor-1 and -2 Derived Peptides Inhibit Proteinase-Activated Receptor-2 Activation by Trypsin

BAHJAT AL-ANI, MAHMOUD SAIFEDDINE, SURANGA J. WIJESURIYA and MORLEY D. HOLLENBERG

Diabetes and Endocrine Research Group, Department of Pharmacology and Therapeutics (B.A.-A., M.S., S.J.W., M.D.H.), and Department of Medicine (M.D.H.), University of Calgary, Faculty of Medicine, Calgary, Alberta, Canada

Received August 29, 2001; accepted November 2, 2001 This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

Trypsin activates proteinase-activated receptor-2 (PAR2) by a mechanism that involves the release of a tethered receptor-activating sequence. We have identified two peptides, FSL-LRY-NH2 (FSY-NH2) and LSIGRL-NH2 (LS-NH2) that block the ability of trypsin to activate PAR2 in a cell line expressing human embryonic kidney cells. Half-maximal inhibition (IC50) by FSY-NH2 and LS-NH2 of the activation of PAR2 by trypsin in a PAR2-expressing cell line was observed at about 50 and 200 μM, respectively. In contrast, the activation of PAR2 by the PAR2-activating peptide, SLIGRL-NH2 (SL-NH2) was not inhibited by FSY-NH2, LS-NH2, or LRG-NH2. In a casein proteolysis assay, neither FSY-NH2 nor LS-NH2 inhibited the proteolytic action of trypsin on its substrate. In addition, FSY-NH2 and LS-NH2 were unable to prevent trypsin from hydrolyzing a 20-amino acid peptide, GPNSKGR/SLIGRLDTPYGGC representing the trypsin cleavage/activation site of rat PAR2. Similarly, FSY-NH2 and LS-NH2 failed to block the ability of trypsin to release the PAR2 N-terminal epitope that is cleaved from the receptor upon proteolytic activation of receptor-expressing KNRK cells. We conclude that the peptides FSY-NH2 and LS-NH2 block the ability of trypsin to activate PAR2 by a mechanism that does not involve a simple inhibition of trypsin proteolytic activity, but possibly by interacting with a tethered ligand receptor-docking site.

The serine proteinase, trypsin, that acts as a digestive enzyme can also regulate target tissues via the proteolytic activation of a G-protein coupled proteinase-activated receptor, PAR2 (Nystedt et al., 1994; Dery et al., 1998). PAR2 activation by trypsin, like the activation of PAR1 by thrombin, involves the proteolytic unmasking of an amino terminal receptor sequence that acts as a tethered ligand (Vu et al., 1991). As was discovered for the related thrombin-activated receptor PAR1 (Vu et al., 1991), for PAR2 short synthetic peptides based on the proteolytically revealed tethered ligand sequence, beginning with serine in rat (SLIGRL-NH2) and human (SLIGKV-NH2) PAR2, can on their own activate PAR2, so as to mimic the action of trypsin in a variety of tissues and cultured cells (Nystedt et al., 1994; Al-Ani et al., 1995; Hollenberg et al., 1997; Saifeddine et al., 1998). In addition, the peptide, SFLLR-NH2, based on the proteolytically revealed human PAR2 tethered ligand that activates PAR2 was also found to activate PAR2, whereas the partial reverse PAR2 and PAR2-derived peptide sequences, FSL-LRY-NH2 (FSY-NH2) and LSIGRL-NH2 (LS-NH2) failed to activate the PAR2 receptor (Blackhart et al., 1996; Al-Ani et al., 1999b). In the past, a peptide, YFLLRNLP, derived from the tethered ligand sequence of human PAR1, was found to be a partial PAR1 receptor agonist, able to antagonize the action of thrombin in human platelets (Rasmussen et al., 1993), but not in cultured human endothelial cells (Kruse et al., 1995). To date, there is no available PAR2 antagonist. Given the cross-reactivity of PAR1-derived peptides with PAR2, we hypothesized that peptide analogs based on either the PAR1 or PAR2-derived tethered ligand might be able to act as antagonists for the PAR2 receptor, in keeping with the ability of YFLLRNLP to antagonize PAR1 (Rasmussen et al., 1993). To test this hypothesis, we synthesized peptide sequences based on the tethered

ABBREVIATIONS: Amino acids are abbreviated by their one-letter code; PAR, proteinase-activated receptor; AP, activating peptides; STI, soya trypsin inhibitor; BS, antibody targeted to the cleavage/activation sequence (GPNSKGR/SLIGRLDTP) of rat PAR2; FSY-NH2, FSSLRY-NH2; HEK, human embryonic kidney; KNRK, Kirsten virus-transformed kidney (KNRK) cell line or in a rat aorta ring preparation. The reverse PAR2 peptide, LRGILS-NH2 (LRG-NH2) did not do so and FSY-NH2 failed to block trypsin activation of PAR1 in the aorta ring or in PAR1-expressing human embryonic kidney cells. Half-maximal inhibition (IC50) by FSY-NH2 and LS-NH2 of the activation of PAR2 by trypsin in a PAR2-expressing cell line was observed at about 50 and 200 μM, respectively. In contrast, the activation of PAR2 by the PAR2-activating peptide, SLIGRL-NH2 (SL-NH2) was not inhibited by FSY-NH2, LS-NH2, or LRG-NH2. In a casein proteolysis assay, neither FSY-NH2 nor LS-NH2 inhibited the proteolytic action of trypsin on its substrate. In addition, FSY-NH2 and LS-NH2 were unable to prevent trypsin from hydrolyzing a 20-amino acid peptide, GPNSKGR/SLIGRLDTPYGGC representing the trypsin cleavage/activation site of rat PAR2. Similarly, FSY-NH2 and LS-NH2 failed to block the ability of trypsin to release the PAR2 N-terminal epitope that is cleaved from the receptor upon proteolytic activation of receptor-expressing KNRK cells. We conclude that the peptides FSY-NH2 and LS-NH2 block the ability of trypsin to activate PAR2 by a mechanism that does not involve a simple inhibition of trypsin proteolytic activity, but possibly by interacting with a tethered ligand receptor-docking site.

The serine proteinase, trypsin, that acts as a digestive enzyme can also regulate target tissues via the proteolytic activation of a G-protein coupled proteinase-activated receptor, PAR2 (Nystedt et al., 1994; Dery et al., 1998). PAR2 activation by trypsin, like the activation of PAR1 by thrombin, involves the proteolytic unmasking of an amino terminal receptor sequence that acts as a tethered ligand (Vu et al., 1991). As was discovered for the related thrombin-activated receptor PAR1 (Vu et al., 1991), for PAR2 short synthetic peptides based on the proteolytically revealed tethered ligand sequence, beginning with serine in rat (SLIGRL-NH2) and human (SLIGKV-NH2) PAR2, can on their own activate PAR2, so as to mimic the action of trypsin in a variety of tissues and cultured cells (Nystedt et al., 1994; Al-Ani et al., 1995; Hollenberg et al., 1997; Saifeddine et al., 1998). In addition, the peptide, SFLLR-NH2, based on the proteolytically revealed human PAR2 tethered ligand that activates PAR2 was also found to activate PAR2, whereas the partial reverse PAR2 and PAR2-derived peptide sequences, FSL-LRY-NH2 (FSY-NH2) and LSIGRL-NH2 (LS-NH2) failed to activate the PAR2 receptor (Blackhart et al., 1996; Al-Ani et al., 1999b). In the past, a peptide, YFLLRNLP, derived from the tethered ligand sequence of human PAR1, was found to be a partial PAR1 receptor agonist, able to antagonize the action of thrombin in human platelets (Rasmussen et al., 1993), but not in cultured human endothelial cells (Kruse et al., 1995). To date, there is no available PAR2 antagonist. Given the cross-reactivity of PAR1-derived peptides with PAR2, we hypothesized that peptide analogs based on either the PAR1 or PAR2-derived tethered ligand might be able to act as antagonists for the PAR2 receptor, in keeping with the ability of YFLLRNLP to antagonize PAR1 (Rasmussen et al., 1993). To test this hypothesis, we synthesized peptide sequences based on the tethered
lignands of PAR₁ and PAR₂, as described above, with a reverse sequence of the first two amino acids (i.e., FSLLRY-NH₂ and LSIGRL-NH₂). These two peptides were evaluated for their ability to act as PAR₂ antagonists for either trypsin or SLIGRL-NH₂ with a calcium signaling assay method employing rat and human PAR₂-transfected cell lines (Saieddine et al., 1998; Al-Ani et al., 1999a,b) and an endothelium-dependent rat aorta relaxation assay (Hollenberg et al., 1997).

Materials and Methods

PAR₂ Cloning and Expression. Based on the previously determined rat PAR₂ sequence (Saieddine et al., 1998) and in keeping with our previous work (Al-Ani et al., 1999a,b) rat kidney cDNA was prepared using the first-strand cDNA synthesis kit (Pharmacia LKB Biotek, Uppsala, Sweden) according to manufacturer’s recommendations at 37°C for 60 min; 3 µl of this solution was used for polymerase chain reaction (PCR) amplification to prepare a full-length receptor cDNA with primer pairs flanking the entire coding region, designed on the basis of the published rat PAR₂ sequence (Saieddine et al., 1996). The primer pairs were: forward primer, PAR₂ - F: (containing a HindIII site and Kozak sequence shown in bold), 5’ TCAAGCTTCACATGGCGAATCTCCAGCTGGC 3’ and reverse primer, PAR₂ R: (containing Smal site shown in bold) 5’ CCCCCGGTCTAGGATTATTACAC3’. Routinely, amplification was done using 2.5 units of Taq DNA polymerase (Promega, Madison, WI) in a 10 mM Tris-HCl buffer, pH 9.0 (50 µl, final volume) containing 1.5 mM MgCl₂, 50 mM KCl, 0.1% (v/v) Triton X-100, and 0.2 mM concentration of each dNTPs. Amplification was allowed to proceed for 35 cycles beginning with a 1-min denaturing period at 94°C, followed by a 1-min reannealing time at 55°C, and a primer extension period of 2 min at 72°C. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. The PCR product was “gene-cleaned” (Magic PCR Purisa DNA purification system; Promega) and ligated (Ready To Go PCR ligase; Amersham Biosciences AB, Uppsala, Sweden) into the PGEM-T vector (Promega). Two microliters of this ligation mixture was used to transform Escherichia coli strain DH5α to produce permanent clones for both manual and automated sequencing by the dideoxynucleotide chain termination method (Sanger et al., 1977), employing a T7 DNA sequencing kit (Amersham Biosciences, Piscataway, NJ) or via the DNA Services Facility at the University of California, Davis aimed at the sequence of cDNA of interest. Rat PAR₂ cDNA was further subcloned into the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA). Rat PAR₂ was then transfected into Kirsten rat fibroblasts (KRKK cells) (American Type Culture Collection, Manassas, VA). Cells were transfected using the LipofectAMINE method, according to the manufacturer’s instructions (Invitrogen) with 5 µg of each construct used per KRKK cell monolayer (60-mm² flask, 50–70% confluent). Transfected cells (either vector alone or PAR₂-containing vector) were subcloned in geneticin-containing medium (0.6 mg/ml), and PAR₂-expressing cells were isolated by fluorescence-activated cell sorting with the use of the anti-receptor B5 antibody (Al-Ani et al., 1999b) for rat PAR₂, to yield permanent cell lines in which >95% of the populations were found to exhibit reactivity with the antibody. Cells were routinely propagated in geneticin (0.6 mg/ml)-containing Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) fetal calf serum, using 80 cm² plastic T-flasks. Cells were subcultured by resuspension in calcium-free isoscentic saline/EDTA solution, without the use of trypsin. A human PAR₂ construct (Bohm et al., 1996), kindly provided by Dr. N. Bunnell (University of California, San Francisco, CA) was transfected into KRKK cells as described above for rat PAR₂.

Measurement of Calcium Signaling Using Fluorescence Emission. Cells to be used for measurements of trypsin and peptide-stimulated fluorescence emission (reflecting an increase in intracellular calcium) were grown at 37°C in 80 cm² T-flasks under an atmosphere of 5% CO₂ in room air to about 85% confluency and were disaggregated with calcium-free isoscentic phosphate-buffered saline containing 0.2 mM EDTA. Either KNRK cells (wild-type, vector-transfected, or PAR₂-transfected cell lines, as above) or human embryonic kidney (HEK) cells (endothogenously expressing both PAR₁ and PAR₂) were used essentially according to previous described procedures (Kawabata et al., 1999). Disaggregated cells were pelleted by centrifugation and resuspended in 1 ml Dulbecco’s modified Eagle’s medium/10% fetal calf serum for loading with the intracellular calcium indicator, Fluo-3 (Molecular Probes Inc., Eugene, OR) at a final concentration of 22 µM (25 µg/ml) of Fluoro-3 acetoxyethyl ester. Indicator uptake was established in the presence of 0.25 mM sulfipyrazine, over 20 to 25 min at room temperature, after which time cells were washed twice by centrifugation and resuspension with the buffer described below, to remove excess dye. Fluo-3-loaded cells were then resuspended to yield a stock solution (about 6 × 10⁶ cells ml⁻¹) in a buffer (pH 7.4) of the following composition: 150 mM NaCl, 3 mM KCl, 1.5 mM CaCl₂, 20 mM HEPES, 10 mM glucose, and 0.25 mM sulfipyrazone. Fluorescence measurements, reflecting elevations of intracellular calcium, were conducted at 24°C using a PerkinElmer fluorescence spectrometer (PerkinElmer Instruments, Norwalk, CT) with an excitation wavelength of 480 nm and an emission recorded at 530 nm. Cells (about 2 ml of approximately 3 x 10⁶ cells ml⁻¹) were maintained in suspension in a stirred (magnetic flea bar) thermostated plastic cuvette (total volume, 4 ml) and peptide or trypsin stock solutions were added directly to the suspension to monitor agonist-induced changes in fluorescence. The fluorescence signals caused by the addition of test agonists (trypsin or PAR₂ APs) were compared with the fluorescence peak height yielded by replicate cell suspensions treated with 2 µM ionophore A23187 (Sigma Chemical Co., St. Louis, MO). This concentration of A23187 was at the plateau of its concentration-response curve for a fluorescence response. In this assay, KNRK cells expressing either rat or human PAR₂ yielded a robust calcium signal in response to 10 nM trypsin and 10 µM SLIGRL-NH₂ whereas vector-transfected cells lacking PAR₂ do not respond. Measurements were done using three or more replicate cell suspensions derived from two or more independently grown crops of cells. Values in the figure represent the average ± S.E.M. (bars).

Bioassy Procedure. The endothelium-intact rat aorta ring assay used to monitor PAR₂ activation was essentially the same as the one used previously to evaluate the actions of PAR₂-activating peptides (Al-Ani et al., 1995; Hollenberg et al., 1997). Male albino Sprague-Dawley rats (250 to 300 g), cared for in accordance with the guidelines of the Canadian Council on animal care, were sacrificed by cervical dislocation and were immediately anticoagulated by the injection of heparin (1000 units in 2 ml of isotonic saline) into the left ventricular circulation. Clot-free portions of aorta were disected free of adhering tissue and endothelium-intact ring preparation (approximately 2 mm x 2 mm) were cut for use in the bioassy. Aorta ring tissue was equilibrated for 1 h at 37°C in a gassed (5% CO₂, 95% O₂) Krebs-Henseleit buffer, pH 7.4, of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, and 10 mM glucose. As previously described (Al-Ani et al., 1995), the relaxant actions of the PAR₂-activating peptide (PAR₂ AP) SLIGRL-NH₂ and trypsin (10 nM) at a concentration that does not activate PAR₁ (Vu et al., 1991) were measured in endothelial-intact rat aorta rings that were preconstricted with 1 µM phenylephrine. The presence of an intact endothelium was verified by monitoring relaxant response to 1 µM acetylcholine. Agonists and peptide antagonists were added directly to the organ bath (4-ml cuvette) and ring tension was monitored using either Grass or Statham force-displacement transducers.

Assay of Proteolytic Activity of Trypsin. The universal pro tease substrate, resorufin-labeled casein, was used to detect trypsin proteolytic activity, according to the manufacturer’s instructions (Roche Molecular Biochemicals, Summerville, NJ). In brief, 10 nM trypsin was incubated at 37°C for 10 to 60 min with the substrate...
(200 μg in 200 μl) in the presence or absence of 200 μM test inhibitor peptides. The absorbance of the released resorufin, reflecting proteolytic activity, was measured using a Beckman DU 640B Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) at 574 nm.

Measurements of the Proteolytic Release by Trypsin of a PAR2-Activating Peptide from an Inactive Synthetic Peptide Precursor. The peptide sequence: GPNSKGR/SLIGRLDTP-YGGC (P20) represents the trypsin cleavage (site denoted by /) — activation site (active tethered ligand shown in bold) of rat PAR2. The sequence YGGC was added for radiolabeling and cysteine-linked protein conjugation. After trypsin cleavage, the sequence, SLIGRLDTP, . . . becomes a receptor activating ligand. P20 itself does not activate PAR2. The ability of trypsin (2 nM) to release the receptor-activating sequence, SLIGRLDTP from P20 in the absence or presence of other peptides (FSSLRY-NH2, LSIGRL-NH2; each at 200 μM) was tested using the following protocol. First, trypsin (2 nM) in the absence or presence of 200 μM FSSLRY-NH2 or LSIGRL-NH2 was incubated with P20 (20 μM) for 3 min at room temperature, at which point the proteolytic reaction was quenched by supplementing the solution with added soya trypsin inhibitor (STI: 1 μg ml−1). One minute thereafter, the STI-quenched reaction mixture was added to an indicator suspension of rat PAR2-expressing KNRK cells that had been loaded with fluo-3 for monitoring PAR2-induced elevations of intracellular calcium, according to the method described above. The generation of a calcium signal (E530) by trypsin treatment of P20, followed by the addition of STI, indicated the successful unmasking by trypsin cleavage of the receptor-activating sequence (SLIGRLDTP, . . . ) from P20. The calcium signal (E530) response of the cells to the trypsin-cleaved P20 product was expressed as a percentage (% trypsin) of the calcium signal observed in response to 2 nM trypsin that had not been incubated either with peptides or STI. As a control, STI (1 μg ml−1) was added to the peptide substrate solution prior to the addition of trypsin, in which case no subsequent calcium signal was generated, indicating a lack of P20 cleavage.

Peptides and Other Reagents. All peptides were synthesized by solid-phase methods at the peptide synthesis facility, University of Calgary, Faculty of Medicine (Calgary, AB, Canada; director, Dr. Denis McMaster). High-performance liquid chromatography analysis, mass spectral analysis, and quantitative amino acid analysis confirmed the composition and purity of all peptides. Stock solutions, prepared in 25 mM of HEPES buffer, pH 7.4, were standardized by quantitative amino acid analysis to verify peptide concentration and purity. Porcine trypsin (14,900 units mg−1) was obtained from Sigma Chemical Co. A maximum specific activity of 20,000 units mg−1 was used to calculate the approximate molar concentration of trypsin in the incubation medium (1U ml−1 = 2 nM). Human thrombin (3186 units/mg protein) was from Calbiochem (San Diego, CA); a concentration of 1 unit/ml was calculated to be approximately 10 nM.

Monitoring Trypsin Removal of the PAR2 N-terminal Epitope in Intact Cells by Immunocytochemistry. The rat PAR2 clone used for the receptor-expressing KNRK cell line possesses an N-terminal sequence that is proximal to the receptor’s cleavage/activation sequence and that is therefore released from the cell upon trypsin activation of PAR2. We generated a rabbit antiserum (SLAW) targeted to an antigenic epitope (SLAWLIGPN-SKGR-GGYGGC) (epitope represented by bold) in the proteinase-release sequence. The polyclonal antiserum (SLAW) was raised in rabbits as described elsewhere (Kong et al., 1997; Al-Ani et al., 1999b) for a B5 anti-PAR2 polyclonal antibody used by us previously (Kong et al., 1997; Al-Ani et al., 1999b). The B5 antiserum recognizes the PAR2 receptor cleavage/activation sequence (GPNSKGR/SLIGRLDTP) and can recognize both the cleaved/activated receptors as well as the uncleaved receptor. Neither the B5 nor the SLAW antibodies react with KNRK cells transfected with vector alone and the reactivity of both antibodies with PAR2-expressing KNRK cells was abolished by preabsorption with the immunizing peptide (e.g., see Al-Ani et al., 1999b for B5 antibody and Fig. 7).

The SLAW antiserum was employed with an immunohistochemical approach to demonstrate a loss of the N-terminal precleavage epitope upon proteolytic activation of PAR2 (Compton et al., 2001). PAR2-expressing cells bearing the N-terminal epitope were or were not treated with trypsin (10 nM) in the absence or presence of FSSLRY-NH2 (200 μM) for 3 min at room temperature at which time soya trypsin inhibitor (1 μg/ml) was added to terminate proteolysis. Cell surface epitope was then detected using cytopsins of treated cells, prepared with a Shandon cytopsin (Shandon Scientific, Cheshire, England) followed by fixation with 95% ethanol. Cell surface receptor was visualized with a 3,3’-diaminobenzidine substrate immunocytochemistry protocol utilized by us previously and described in detail elsewhere (Saifeddine et al., 2001). The removal of the N-terminal epitope by trypsin was visualized by a disappearance of cell surface reactivity with the SLAW antibody; receptor cleaved at the cell surface, but internalized in the trypsin-treated cells was detected with the B5 antiserum.

Results

FSSLRY-NH2 and LSIGRL-NH2 Block Trypsin, but Not SLIGRL-NH2 Activation of PAR2 in Receptor-Expressing KNRK Cells. We first tested the ability of FSSLRY-NH2 (FSY-NH2) and LSIGRL-NH2 (LS-NH2) to affect calcium signaling in KNRK cells expressing rat PAR2 (Fig. 1).
Comparable results were obtained with cells expressing human PAR2 (data not shown and Fig. 2). Although preincubation of the PAR2-KNRK cells with the complete reverse PAR2AP, LRGILS-NH2 (LRG-NH2, 200 μM: tracing A, Fig. 1), had no effect on the magnitude of the calcium signal elicited by 2 nM trypsin, both FSY-NH2 (200 μM: tracing B, Fig. 1) and LS-NH2 (200 μM: tracing C, Fig. 1) inhibited the trypsin-triggered response by at least 70%. In contrast, neither FSY-NH2 (tracing D, Fig. 1) nor LS-NH2 and LRG-NH2 (identical to tracing D, Fig. 1, not shown) at a concentration of 200 μM affected the calcium response caused by the PAR2AP, SLIGRL-NH2 (SL-NH2, 3 μM). Also, at a higher concentration (400 μM), FSY-NH2 and LS-NH2 failed to cause significant inhibition of the calcium signal triggered by 3 μM SL-NH2 (data not shown). The concentrations of trypsin and SL-NH2 used were at the EC50 concentrations of their respective concentration-effect curves, causing a calcium signal equivalent to that of the test ionophore, A23187 (2 μM, Fig. 1, right-hand tracings) (Vergnolle et al., 1998 and data not shown). In addition, calcium responses were not detected in response to trypsin (20 nM) or SL-NH2 (50 μM) either in the “empty” vector-transfected KNRK cell line or in a nontransfected background KNRK cell line (Al-Ani et al., 1999b and data not shown).

To assess the relative potencies with which FSY-NH2 and LS-NH2 were able to inhibit the trypsin-activated PAR2 signal for both rat and human PAR2, concentration-inhibition measurements were done for increasing concentrations of the inhibitor peptides at a constant concentration of trypsin (2 nM for rat PAR2; 10 nM for human PAR2; Fig. 2). In the assay, the inhibitory potency of FSY-NH2 (IC50 = 50 μM) was about 4-fold greater than that of LS-NH2 (IC50 = 200 μM).

We next wanted to investigate whether the PAR2 derived peptide (FSLLRY-NH2) was able to block thrombin-induced cellular responses via PAR1, similar to the above demonstrated inhibition of trypsin-induced activation of PAR2. The HEK 293 cell line that expresses both functional PAR1 and PAR2 (Kawabata et al., 1999) was used to test this hypothesis. In the HEK cells, thrombin activates only PAR1, whereas trypsin, at concentrations lower than 25 nM, activates only PAR2 (Kawabata et al., 1999). In the absence of FSY-NH2 peptide, trypsin caused a robust calcium signal via PAR2 activation in the same cell suspension that had previously responded to thrombin (Fig. 3A). The response to trypsin was the same as in cells that had not been previously activated by thrombin (data not shown). As shown in Fig. 3B, the addition of FSY-NH2 (200 μM) failed to prevent thrombin (20 nM) from activating PAR1. In contrast, the peptide blocked the activation of PAR2 by trypsin in the same cell suspension that had responded to thrombin (Fig. 3B).

FSLLRY-NH2 and LSIGRL-NH2 Blocked the Relaxant Action of Trypsin but Not SLIGRL-NH2 in the Aorta Ring Bioassay. In view of the ability of FSY-NH2 and LS-NH2 to block trypsin-mediated calcium signaling in human and rat PAR2 expressing KNRK cells, we hypothesized that in the rat aorta bioassay, the peptides might also block the relaxant action of trypsin, which is mediated via endothelial PAR2 (Al-Ani et al., 1995; Saieddine et al., 1996). As shown in Fig. 4, the relaxant action of trypsin, at a concentration (1 nM), well below that which might activate PAR1, was essentially eliminated in the presence of 200 μM FSY-NH2, whereas the relaxant action of the PAR2-activating peptide, SL-NH2, was not affected (Fig. 4A). At a concentration of 400 μM, LS-NH2 reduced the relaxant effect of trypsin by about 50% (Fig. 4B), but like FSY-NH2, had no effect on relaxation caused by SL-NH2 (data not shown). In addition, there was no inhibition of the relaxant action of thrombin (200 nM) by 200 μM of the peptide FSY-NH2 (Fig. 4C). This concentration of FSY-NH2 markedly suppressed the response
The Proteolytic Activity of Trypsin Is Not Affected by FSLLR-NH₂ and SLIGRL-NH₂. One key issue to consider was that the ability of FSY-NH₂ to inhibit thrombin action, the action of LS-NH₂ to affect the activation of PAR₂ by trypsin might be due simply to an inhibitory action on the proteolytic activity of trypsin (e.g., see Bhattacharya et al., 2001). This possibility was tested in three ways. First, the ability of FSY-NH₂ and LS-NH₂ to reduce the proteolytic activity of trypsin was tested in a nonspecific proteolysis assay, using resorufin-labeled casein as a substrate. Second, trypsin in the presence and absence of either FSY-NH₂ or LS-NH₂, was evaluated for its ability to hydrolyze a synthetic peptide substrate, GPNSKGR/SLIGRLDVP (P20) representing the cleavage/activation sequence of rat PAR₂. Third, in the presence and absence of either FSY-NH₂ or LS-NH₂, the ability of trypsin to cleave and release the N-terminal portion of PAR₂, that is proximal to the receptor cleavage/activation site, was evaluated in KNRK cells expressing rat PAR₂ with its N-terminal epitope detected with the SLAW antiserum. As shown in Fig. 5, the rate of proteolysis of resorufin-labeled casein by 10 nM trypsin was not altered in the presence of either FSY-NH₂ or LS-NH₂ at the same concentrations (200 μM) that inhibited PAR₂ activity in the cell and tissue assays (Figs. 1–4). Also, FSY-NH₂ and LS-NH₂ did not affect trypsin-mediated casein hydrolysis by 2 nM trypsin over a 30-min time period (not shown). As shown in Fig. 6, brief (3 min) trypsin treatment at room temperature of the peptide (P20) representing the activation/cleavage sequence of rat PAR₂ (GPNSKGR/SLIGRLDVP) followed by quenching of the reaction with soya trypsin inhibitor, yielded a proteolysis product (presumably, SLIGRL... ) capable of activating PAR₂ (Fig. 6, A and B). In keeping with the results of the casein proteolysis experiment, the ability of trypsin (2–10 nM) to cleave P20 to reveal its PAR₂-activating sequence was not affected in the presence of either FSY-NH₂ or LS-NH₂ at a concentration (200 μM) that inhibited trypsin activation of PAR₂ in the calcium-signaling assay. In contrast, the addition of soya trypsin inhibitor to the P20 substrate solution prior to the addition of trypsin completely abolished the release of a PAR₂ activating peptide (no calcium signal, Fig. 6A, third histogram from left; Fig. 6B, top tracing, middle response). Finally, we wanted to investigate
whether FSY-NH₂ and LS-NH₂ interfered with the ability of trypsin to release the amino terminal SLAW epitope from PAR₂ by proteolytic activation. As shown in Fig. 7, either in the absence (Fig. 7A, Trp; Fig. 7D) or presence of either FSY-NH₂ (Fig. 7A, FSY-NH₂ + Trp; Fig. 7E) or LS-NH₂ (Fig. 7A, LS-NH₂ + Trp; Fig. 7F), trypsin proteolysis was essentially equally effective in removing the PAR₂ epitope, that is N-terminal to the receptor cleavage/activation site equivalent to P20. The untreated PAR₂ expressing cells (Fig. 7A, Control; Fig. 7B) had positive immunoreactivity (granular cell membrane staining) with the SLAW anti-receptor antibody. It was also found that adding a premixed solution of soya trypsin inhibitor and trypsin to the cells prior to the addition of the antibody had no effect on the immunostaining of these cells (data not shown). In addition, immunoreactivity was detected neither when the SLAW antibody was preabsorbed with the receptor-derived peptide immunogen (Fig. 7C) nor in the empty vector-transfected KNRK cell line (data not shown).

Discussion

The main finding for our study was that the PAR₁/PAR₂-related peptides, FSLLRY-NH₂ and LSIGRL-NH₂, while unable on their own to activate PAR₂, were able to block the ability of trypsin to do so, either in a PAR₂ calcium signaling

![Image](https://via.placeholder.com/150)

**Fig. 6.** Proteolytic cleavage by trypsin of a peptide PNSGR/SLIGRLTPGCGC (P20) representing the cleavage/activation domain of rat PAR₂ to yield PAR₂-activating peptide: lack of effect of FSY-NH₂ and LS-NH₂ and inhibition by soy trypsin inhibitor. As outlined under Materials and Methods, P20 (20 μM) was incubated with 2 mM trypsin at room temperature for 3 min. in the absence or presence of FSY-NH₂ (200 μM), LS-NH₂ (200 μM), or soya trypsin inhibitor (STI 1 μg/ml), at which point the reaction was quenched by the addition of added STI (1 μg/ml). One minute thereafter, the reaction mixture was added to an indicator cell suspension of fluo-3-loaded rat PAR₂ KNRK cells, and the calcium signal generated was monitored. The calcium signal caused by the release of PAR₂ activating peptide from P20 was compared with the "control" signal generated by trypsin alone. A, histograms show the average calcium signals (± S.E.M., bars: % control trypsin response; open histogram) caused by the release of a PAR₂-activating peptide from P20. On its own, P20 did not elicit a calcium response (second histogram from left). Although the addition of STI prior to the addition of trypsin abolished the ability of trypsin to release a PAR₂-activating peptide from P20 (third histogram from left), the addition of STI after trypsin was combined with P20 did not affect the subsequent calcium signal (fourth histogram from left). Neither FSY-NH₂ nor LS-NH₂ affected the trypsin-mediated release of PAR₂-activating peptide from P20 to cause a calcium signal (last two histograms on right). B, representative tracings of calcium signals. Each tracing shows the calcium signal generated in fluo-3-loaded rat PAR₂ KNRK cells by the P20 proteolytic product released by trypsin in the absence or presence of 200 μM added peptide (FSY-NH₂, tracing (b); LS-NH₂, tracing (c)) or STI (1 μg/ml: tracing (a)). The order of addition of P20 peptide, STI, LS-NH₂, FSY-NH₂, and trypsin are shown at the bottom of each tracing. The control responses to trypsin are shown on the right.

![Image](https://via.placeholder.com/150)

**Fig. 7.** Cleavage of PAR₂ N-terminal SLAW epitope by trypsin in the presence of FSY-NH₂ and LS-NH₂. As outlined under Materials and Methods, permanently transfected PAR₂ expressing KNRK cells were treated with trypsin alone (A, Trp and D), or trypsin in the presence of FSY-NH₂ (A, FSY-NH₂ + Trp and E) or LS-NH₂ (A, LS-NH₂ + Trp and F) and subjected to cytospin followed by immunocytochemistry with the SLAW antiserum. Positive control: cells were treated with the vehicle (A, Control and B). Negative control: cells were treated with the vehicle and the SLAW antibody preabsorbed by the immunizing peptide (C). The arrows (in B–F) indicate presence (B) or absence (C–F) of granular cell membrane immunoreactivity of the SLAW antibody. The bar in B represents 25 μm. In A, the histograms represent the percentage of average positive cells for 10 fields (approximately 700 examined cells) per slide. Error bars represent ± S.E.M.
assay using PAR2-expressing KN92 cells (Saifeddine et al., 1998; Al-Ani et al., 1999b) or in intact aorta tissue (Fig. 4) wherein proteolytic activation of PAR2 causes an endothelium-dependent nitric oxide-mediated vasorelaxation (Al-Ani et al., 1995; Saifeddine et al., 1996). Notwithstanding, neither of these two peptides that antagonize trypsin-mediated PAR2 activation affected receptor triggering by 100 to 400-fold lower concentrations of the PAR2-activating peptide, SLIGRL-NH2 (Fig. 1, tracing D and Fig. 4). One possible hypothesis that we considered for the ability of FSY-NH2 and LS-NH2 to block trypsin-mediated activation of PAR2 without affecting activation caused by SL-NH2 was that the peptides were simply inhibiting directly the proteolytic activity of trypsin, as did STI, to prevent the cleavage/unmasking of the cell-attached tethered ligand (e.g., see Bhattacharya et al., 2001). However, the results of three independent approaches to test this hypothesis indicated that the peptides, at concentrations that blocked trypsin (2–10 nM)-mediated PAR2 activation, did not affect trypsin’s proteolytic activity in the presence of the peptides at 200 μM: 1) the rate of hydrolysis of resorufin-labeled casein was unaffected, 2) cleavage of P20 to yield its PAR2-activating sequence was unaffected, and 3) the ability of trypsin acting on receptor-expressing cells to strip the PAR2 epitope, N-terminal to the receptor cleavage/activation site, was not impeded.

We therefore conclude that a mechanism other than a direct inhibition of trypsin proteolytic activity was responsible for the inhibitory effects of the peptides. This conclusion is supported further by the inability of FSY-NH2 to inhibit activation of PAR2 by the serine proteinase thrombin. A working hypothesis we suggest is that although the peptides FSY-NH2 and LS-NH2 do not block the ability of trypsin to unmask the tethered receptor-activating ligand sequence, SLIGRL-NH2 . . . that they are, nonetheless, able to impede access of the proteolytically revealed tethered ligand to the receptor-activating site, whereas the same peptides do not block access of the soluble receptor-activating peptides to distinct but possibly overlapping receptor activation sites in the extracellular receptor loops. The sites on PAR2 at which FSY-NH2 interacts would appear to be receptor-specific, in that the same peptide did not affect the ability of the PAR2 tethered ligand revealed by thrombin to activate PAR1. This possible difference between the docking sites of the soluble and tethered ligand in PAR2 would be in keeping with data obtained for human PAR1 (Blackhart et al., 2000), demonstrating differences between soluble receptor-activating ligands and the protease-revealed tethered ligand for activating selected PAR2 receptor mutants. Furthermore, our own work with a mutated PAR2 receptor having arginines substituted for glutamatic acid in extracellular loop 2 showed that the tryptic revealed tethered ligand (SLIGRL-NH2 . . . was more effective in activating the receptor than was the comparable soluble receptor-activating ligand, SLIGRL-NH2 (Al-Ani et al., 1999a). Taken together, the data obtained by us for PAR2 (Al-Ani et al., 1999a) and for PAR1 by Blackhart et al., (2000) point to different receptor activation sites for the tethered and soluble ligands. Such differences, we believe, could conceivably account for the differential ability of FSY-NH2 and LS-NH2 to block trypsin but not SL-NH2-mediated activation of PAR2. Although further work will be required to establish the molecular basis for the suggested differences between the interactions of the tethered and soluble ligands with the receptor, our data obtained with FSY-NH2 and LS-NH2 suggest that any future research for putative PAR2 receptor antagonists should take both potential mechanisms into account, so as to assess receptor activation both by proteolysis with tryptic and by a receptor-activating peptide such as SL-NH2.

Acknowledgments

We are grateful to Dr. Nigel Bunnett for providing the human PAR2 clone. We thank Joanne Forden for assistance with the proteinase assay and Marilyn Barnes for assistance with manuscript preparation.

References


Address correspondence to: Dr. M. D. Hollenberg, Department of Pharmacology and Therapeutics, University of Calgary Faculty of Medicine, 3330 Hospital Drive N.W., Calgary, AB Canada T2N 4N1. E-mail: mhollenb@ucalgary.ca

Received August 30, 2001. Accepted March 1, 2002.