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

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ABSTRACT

Trypsin activates proteinase-activated receptor-2 (PAR_2) by a mechanism that involves the release of a tethered receptor-activating sequence. We have identified two peptides, FS-LLRY-NH_2 (FSY-NH_2) and LSIGRL-NH_2 (LS-NH_2) that block the ability of trypsin to activate PAR_2 either in PAR_2-expressing Kirsten virus-transformed kidney (KNRK) cell lines or in a rat aorta ring preparation. The reverse PAR_2 peptide, LRGILS-NH_2 (LRG-NH_2) did not do so and FSY-NH_2 failed to block thrombin activation of PAR_1 in the aorta ring or in PAR_1-expressing human embryonic kidney cells. Half-maximal inhibition (IC_{50}) by FSY-NH_2 and LS-NH_2 of the activation of PAR_2 by trypsin in a PAR_2 KNRK calcium-signaling assay was observed at about 50 and 200 μM, respectively. In contrast, the activation of PAR_2 by the PAR_2-activating peptide, SLIGRL-NH_2 (SL-NH_2) was not inhibited by FSY-NH_2, LS-NH_2, or LRG-NH_2. In a casein proteolysis assay, neither FSY-NH_2 nor LS-NH_2 inhibited the proteolytic action of trypsin on its substrate. In addition, FSY-NH_2 and LS-NH_2 were unable to prevent trypsin from hydrolyzing a 20-amino acid peptide, GPNSKGR/SLIGRLDTPYGGC representing the trypsin cleavage/activation site of rat PAR_2. Similarly, FSY-NH_2 and LS-NH_2 failed to block the ability of trypsin to release the PAR_2 N-terminal epitope that is cleaved from the receptor upon proteolytic activation of receptor-expressing KNRK cells. We conclude that the peptides FSY-NH_2 and LS-NH_2 block the ability of trypsin to activate PAR_2 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, PAR_2 (Nystedt et al., 1994: Dery et al., 1998). PAR_2 activation by trypsin, like the activation of PAR_1 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 PAR_1 (Vu et al., 1991), for PAR_2 short synthetic peptides based on the proteolytically revealed tethered ligand sequence, beginning with serine in rat (SLIGRL-NH_2) and human (SLIGKV-NH_2) PAR_2, can on their own activate PAR_2, 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-NH_2, based on the proteolytically revealed human PAR_1 tethered ligand that activates PAR_1, was also found to activate PAR_2, whereas the partial reverse PAR_1 and PAR_2-derived peptide sequences, FS-LLRY-NH_2 (FSY-NH_2) and LSIGRL-NH_2 (LS-NH_2) failed to activate the PAR_2 receptor (Blackhart et al., 1996; Al-Ani et al., 1999b). In the past, a peptide, YFLLRNP, derived from the tethered ligand sequence of human PAR_1, was found to be a partial PAR_1 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 PAR_2 antagonist. Given the cross-reactivity of PAR_1-derived peptides with PAR_2, we hypothesized that peptide analogs based on either the PAR_1 or PAR_2-derived tethered ligand might be able to act as antagonists for the PAR_2 receptor, in keeping with the ability of YFLLRNP to antagonize PAR_1 (Rasmussen et al., 1993). To test this hypothesis, we synthesized peptide sequences based on the tethered

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ABBREVIATIONS: Amino acids are abbreviated by their one-letter code; PAR, proteinase-activated receptor; AP, activating peptides; STI, soya trypsin inhibitor; B5, antibody targeted to the cleavage/activation sequence (GPNSKGRSLIGRLDTP) of rat PAR_2; FSY-NH_2, FSLLRY-NH_2; HEK, human embryonic kidney; KNRK, Kirsten virus-transformed rat kidney; LRG-NH_2, LRGILS-NH_2; LS-NH_2, LSIGRL-NH_2; P20, GPNSKGRSLIGRLDTPYGGC, peptide representing the PAR_2 cleavage/activation site with a C-terminal sequence (YGGC) added for radiolabeling and protein conjugation via cysteine; PCR, polymerase chain reaction; SLAW, antibody targeted to the N-terminal epitope on PAR_2, released by trypsin; SL-NH_2, SLIGRL-NH_2.

ligands of PAR_1 and PAR_2, as described above, with a reverse sequence of the first two amino acids (i.e., FSLLRY-NH_2 and LSIGRL-NH_2). These two peptides were evaluated for their ability to act as PAR_2 antagonists for either trypsin or SLIGRL-NH_2 with a calcium signaling assay method employing rat and human PAR_2-transfected cell lines (Saifeddine et al., 1998; Al-Ani et al., 1999a,b) and an endothelium-dependent rat aorta relaxation assay (Hollenberg et al., 1997).

Materials and Methods

PAR_2 Cloning and Expression. Based on the previously determined rat PAR_2 sequence (Saifeddine et al., 1996) 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 Biotechnology, 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 PAR_2 sequence (Saifeddine et al., 1996). The primer pairs were: forward primer, PAR_2 - F: (containing a *Hin*dIII site and Kozak sequence shown in bold), 5′ TC**AAGCTTCCACC**ATGCGAAGTCTCAGCCTGGC 3′ and reverse primer, PAR_2 R: (containing *Sma*I site shown in bold) 5′ **CCCGGG**CTCAGTAGGAGGTTTTAACAC3′. 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 $MgCl_2$, 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 Preps DNA purification system; Promega) and ligated (Ready To Go T4 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 Calgary Faculty of Medicine. Then, the rat PAR_2 cDNA was further subcloned into the pcDNA3 mammalian expression vector (Invitrogen, Carlsbad, CA). Rat PAR_2 was then transfected into Kirsten virus-transformed rat kidney (KNRK) 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 KNRK cell monolayer (60-mm^2 flask, 50–70% confluent). Transfected cells (either vector alone or PAR_2-containing vector) were subcloned in geneticin-containing medium (0.6 mg/ml), and PAR_2-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 PAR_2, to yield permanent cell lines in which >95% of the populations were found to exhibit reactivity with the antibody. Cells were routinely propagated in genecitin (0.6 mg/ml)-containing Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal calf serum, using 80 cm^2 plastic T-flasks. Cells were subcultured by resuspension in calcium-free isotonic saline/EDTA solution, without the use of trypsin. A human PAR_2 construct (Bohm et al., 1996), kindly provided by Dr. N. Bunnett (University of California, San Francisco, CA) was transfected into KNRK cells as described above for rat PAR_2.

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 cm^2 T-flasks under an atmosphere of 5% CO_2 in room air to about 85% confluency and were disaggregated with calcium-free isotonic phosphate-buffered saline containing 0.2 mM EDTA. Either KNRK cells (wild-type, vector-transfected, or PAR_2-transfected cell lines, as above) or human embryonic kidney (HEK) cells (endogenously expressing both PAR_1 and PAR_2) were used essentially according to previously 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 $\mu g\ ml^{-1}$) of Fluo-3 acetoxymethyl ester. Indicator uptake was established in the presence of 0.25 mM sulfinpyrazone, 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 6×10^6 cells ml^{-1}) in a buffer (pH 7.4) of the following composition: 150 mM NaCl, 3 mM KCl, 1.5 mM$CaCl_2$, 20 mM HEPES, 10 mM glucose, and 0.25 mM sulfinpyrazone. 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 3×10^5 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 PAR_2APs) 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 PAR_2 yield a robust calcium signal in response to 10 nM trypsin and 10 μM SLIGRL-NH_2, whereas vector-transfected cells lacking PAR_2 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).

Bioassay Procedure. The endothelium-intact rat aorta ring assay used to monitor PAR_2 activation was essentially the same as the one used previously to evaluate the actions of PAR_2-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 dissected free of adhering tissue and endothelium-intact ring preparation (approximately 2 mm × 2 mm) were cut for use in the bioassay. Aorta ring tissue was equilibrated for 1 h at 37°C in a gassed (5% CO_2, 95% O_2) Krebs-Henseleit buffer, pH 7.4, of the following composition: 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 10 mM glucose. As previously described (Al-Ani et al., 1995), the relaxant actions of the PAR_2-activating peptide (PAR_2AP) SLIGRL-NH_2, and trypsin (10 nM) at a concentration that does not activate PAR_1 (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 protease 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.

Measurement of the Proteolytic Release by Trypsin of a PAR_2-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 PAR_2. 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 PAR_2. The ability of trypsin (2 nM) to release the receptor-activating sequence, SLIGRLDTP from P20 in the absence or presence of other peptides (FSLLRY-NH_2, LSIGRL-NH_2: each at 200 μM) was tested using the following protocol. First, trypsin (2 nM) in the absence or presence of 200 μM FSLLRY-NH_2 or LSIGRL-NH_2 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 $\mu g\ ml^{-1}$). One minute thereafter, the STI-quenched reaction mixture was added to an indicator suspension of rat PAR_2-expressing KNRK cells that had been loaded with fluo-3 for monitoring PAR_2-induced elevations of intracellular calcium, according to the method described above. The generation of a calcium signal (E_{530}) by trypsin treatment of P20, followed by the addition of STI, indicated the successful unmasking by trypsin cleavage of the receptor-activating sequence (SLIGRLDP. . .) from P20. The calcium signal (E_{530}) 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 $\mu g\ ml^{-1}$) 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 $ml^{-1} \cong 2$ 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 PAR_2 N-terminal Epitope in Intact Cells by Immunocytochemistry. The rat PAR_2 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 PAR_2. We generated a rabbit antiserum (SLAW) targeted to an antigenic epitope (**SLAWLLGGPNSKGR**-GGYGGC) (epitope represented by bold) in the proteinase-released 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-PAR_2 polyclonal antibody used by us previously (Kong et al., 1997; Al-Ani et al., 1999b). The B5 antiserum recognizes the PAR_2 receptor cleavage/activation sequence (**GPNSKGRSLIGRLDTP**) 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 PAR_2-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 PAR_2 (Compton et al., 2001). PAR_2-expressing cells bearing the N-terminal epitope were or were not treated with trypsin (10 nM) in the absence or presence of FSLLRY-NH_2 (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 cytospins 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

FSLLRY-NH_2 and LSIGRL-NH_2 Block Trypsin, but Not SLIGRL-NH_2 Activation of PAR_2 in Receptor-Expressing KNRK Cells. We first tested the ability of FSLLRY-NH_2 (FSY-NH_2) and LSIGRL-NH_2 (LS-NH_2) to affect calcium signaling in KNRK cells expressing rat PAR_2 (Fig.

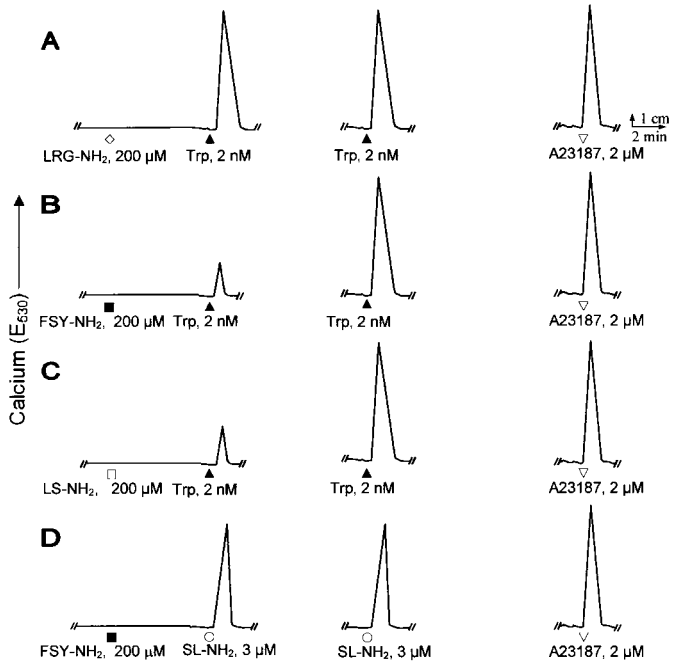

Fig. 1. Differential inhibition by FSLLRY-NH_2 (FSY-NH_2) and LSIGRL-NH_2 (LS-NH_2) of PAR_2 activation by either trypsin or SLIGRL-NH_2 (SL-NH_2). Calcium signaling (E_{530}, reflecting increases in intracellular calcium) in rat PAR_2-KRK cells in response to either trypsin (▲, Trp, 2 nM) or the PAR_2-activating peptide, SL-NH_2 (○, SL-NH_2, 3 μM), either in the absence (middle tracings) or presence (left-hand tracings) of inhibitory peptides was measured as outlined under *Materials and Methods*. A, lack of inhibition of trypsin signaling by the reverse PAR_2-activating peptide, LRGILS-NH_2 (◇, LRG-NH_2, 200 μm). B, inhibition by FSY-NH_2 (■, FSY-NH_2, 200 μM) of trypsin (2 nM) signaling. C, inhibition of trypsin signaling by LS-NH_2 (□, LS-NH_2, 200 μM). D, lack of inhibition of SL-NH_2 (3 μM)-mediated signaling by FSY-NH_2. The control cellular responses to trypsin (▲) and SL-NH_2 (○) are shown in the middle of each set of tracings (A to D) and can be compared with the cellular response to 2 μM calcium ionophore (▽, A23187, 2 μM). The antagonist peptides were added to the cell suspension approximately 6 min prior to the addition of either trypsin (▲, 2 nM) or SL-NH_2 (○, 3 μM). The scale for time and calcium signal is shown to the right of tracing A. The results are representative of 3 or more separately conducted experiments with independently grown crops of rat PAR_2-KNRK cells.

1). Comparable results were obtained with cells expressing human PAR_2 (data not shown and Fig. 2). Although preincubation of the PAR_2-KNRK cells with the complete reverse PAR_2AP, LRGILS-NH_2 (LRG-NH_2, 200 μM: tracing A, Fig. 1), had no effect on the magnitude of the calcium signal elicited by 2 nM trypsin, both FSY-NH_2 (200 μM: tracing B, Fig. 1) and LS-NH_2 (200 μM: tracing C, Fig. 1) inhibited the trypsin-triggered response by at least 70%. In contrast, neither FSY-NH_2 (tracing D, Fig. 1) nor LS-NH_2 and LRG-NH_2 (identical to tracing D, Fig. 1, not shown) at a concentration of 200 μM affected the calcium response caused by the PAR_2AP, SLIGRL-NH_2 (SL-NH_2, 3 μM). Also, at a higher concentration (400 μM), FSY-NH_2 and LS-NH_2 failed to cause significant inhibition of the calcium signal triggered by 3 μM SL-NH_2 (data not shown). The concentrations of trypsin and SL-NH_2 used were at the EC_{50} 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-NH_2 (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-NH_2 and LS-NH_2 were able to inhibit the trypsin-activated PAR_2 signal for both rat and human PAR_2, concentration-inhibition measurements were done for increasing concentrations of the inhibitor peptides at a constant concentration of trypsin (2 nM for rat PAR_2; 10 nM for human PAR_2; Fig. 2). In the assay, the inhibitory potency of FSY-NH_2 ($IC_{50} \cong 50$ μM) was about 4-fold greater than that of LS-NH_2 ($IC_{50} \cong 200$ μM).

We next wanted to investigate whether the PAR_1 derived peptide (FSLLRY-NH_2) was able to block thrombin-induced cellular responses via PAR_1, similar to the above demonstrated inhibition of trypsin-induced activation of PAR_2. The HEK 293 cell line that expresses both functional PAR_1 and PAR_2 (Kawabata et al., 1999) was used to test this hypothesis. In the HEK cells, thrombin activates only PAR_1, whereas trypsin, at concentrations lower than 25 nM, activates only PAR_2 (Kawabata et al., 1999). In the absence of FSY-NH_2 peptide, trypsin caused a robust calcium signal via PAR_2 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-NH_2 (200 μM) failed to prevent thrombin (20 nM) from activating PAR_1. In contrast, the peptide blocked the activation of PAR_2 by trypsin in the same cell suspension that had responded to thrombin (Fig. 3B).

FSLLRY-NH_2 and LSIGRL-NH_2 Blocked the Relaxant Action of Trypsin but Not SLIGRL-NH_2 in the Aorta Ring Bioassay. In view of the ability of FSY-NH_2 and LS-NH_2 to block trypsin-mediated calcium signaling in human and rat PAR_2 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 PAR_2 (Al-Ani et al., 1995; Saifeddine et al., 1996). As shown in Fig. 4, the relaxant action of trypsin, at a concentration (1 nM), well below that which might activate PAR_1, was essentially eliminated in the presence of 200 μM FSY-NH_2, whereas the relaxant action of the PAR_2-activating peptide, SL-NH_2, was not affected (Fig. 4A). At a concentration of 400 μM, LS-NH_2 reduced the relaxant effect of trypsin by about 50% (Fig. 4B), but like FSY-NH_2, had no effect on relaxation caused by SL-NH_2 (data not shown). In addition, there was no inhibition of the relaxant action of thrombin (200 nM) by 200 μM of the peptide FSY-NH_2 (Fig. 4C). This concentration of FSY-NH_2 markedly suppressed the response

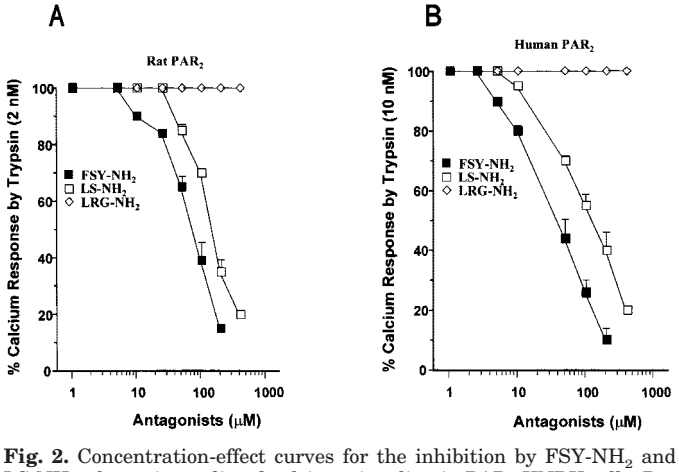

Fig. 2. Concentration-effect curves for the inhibition by FSY-NH_2 and LS-NH_2 of trypsin-mediated calcium signaling in PAR_2-KNRK cells. Rat PAR_2-KNRK (A) and human PAR_2-KNRK (B) cells loaded with fluo-3 as outlined under *Materials and Methods* were or were not treated with increasing concentrations of either FSY-NH_2 (■) or LS-NH_2 (□), and a response to added trypsin (2 nM for rat PAR_2-KNRK; 10 nM for human PAR_2-KNRK) was then measured. Data points represent the mean (± S.E.M., bars) for three or more independent experiments done with triplicate cell suspensions. The responses of peptide-treated cell suspensions were expressed as a percentage (% control) of the control response to trypsin in the absence of added antagonist peptide. No antagonism was observed for the reverse PAR_2-activating peptide, LRGILS-NH_2 (◇, LRG-NH_2, upper curve).

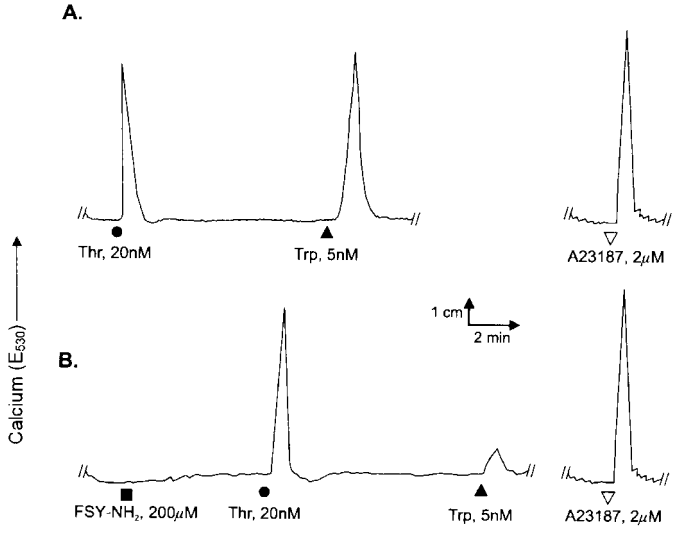

Fig. 3. Inhibition of trypsin but not thrombin mediated calcium signaling by FSY-NH_2 in HEK 293 cells. Calcium signaling (E_{530}), reflecting increases in intracellular calcium was measured in HEK 293 cells in response to thrombin (●, Thr, 20 nM) and trypsin (▲, Trp, 5 nM), either in the absence (A) or presence (B) of FSY-NH_2 (■, FSY-NH_2, 200 μM). Responses were compared relative to the E_{530} signal yielded in each cell sample by 2 μM ionophore, A232187 (▽). The scale for time and calcium signal is shown in the middle. The results are representative of three or more separately conducted experiments with independently grown crops of HEK 293 cells.

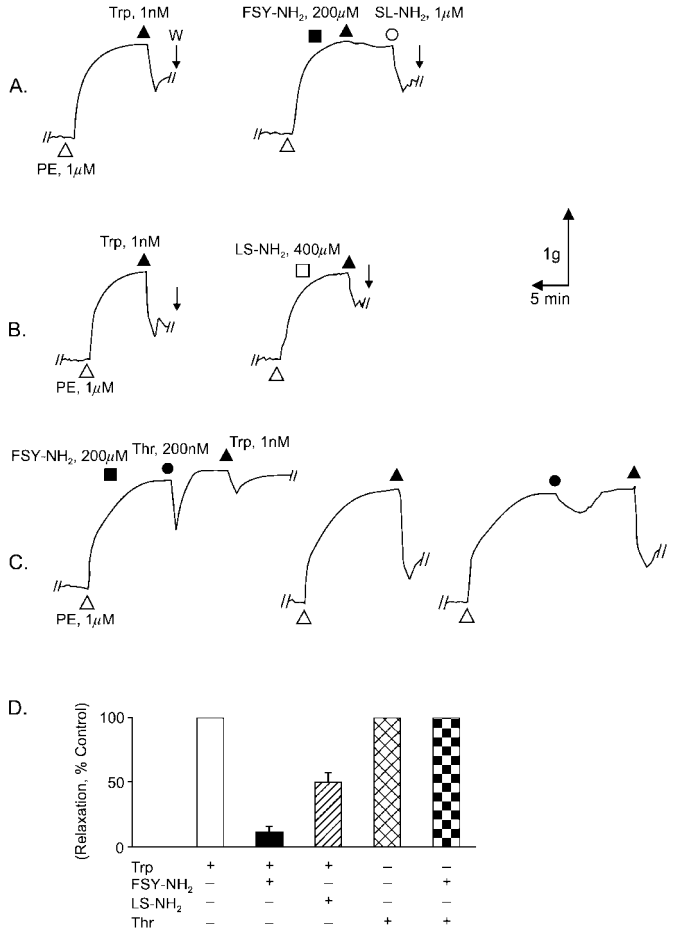

Fig. 4. Inhibition of trypsin but not SL-NH_2 and thrombin mediated relaxation by FSY-NH_2 in the rat aorta ring bioassay. A to C, bioassay tracings; D, histograms showing averaged data (± S.E.M., bars, $n = 3$). Endothelium-intact aorta ring preparations were preconstricted with phenylephrine (△, PE, 1 μM), and the endothelium-dependent relaxant effects of trypsin (▲, Trp, 1 nM), SL-NH_2 (○, 1 μM) or thrombin (●, Thr, 200 nM) were monitored either in the absence (left-hand tracings, A and B; middle tracing, C) or presence of added FSY-NH_2 (■, 200 μM: right tracing, A; left tracing, C) or LS-NH_2 (□, 400 μM; right tracing, B). The control responses to trypsin are shown (on the left, A and B, and in the middle of C). W (arrow) indicates tissue wash. The scale for time and tension in the tissue bioassay is shown to the right of the tracing B. The histograms at the bottom summarize the responses to trypsin (+) and thrombin (+) either in the absence (−) or presence (+) of added peptide (additions shown at bottom).

to trypsin (left-hand tracing, Fig. 4C) in a preparation that had previously responded to thrombin. In the absence of FSY-NH_2, the relaxant response to trypsin in a preparation that had been previously activated by thrombin (right-hand tracing, Fig. 4C) was the same as in a preparation that had not been previously exposed to thrombin (middle tracing, Fig. 4C). Activation of PAR1 by lower concentrations of thrombin (50 or 100 nM) was similarly not affected by 200 μM FSY-NH_2 (data not shown). The histograms in Fig. 4D summarize the actions of FSY-NH_2 and LS-NH_2 on the function of the proteases, trypsin and thrombin. Since FSY-NH_2 failed to inhibit thrombin action, the action of LS-NH_2 was not tested.

The Proteolytic Activity of Trypsin Is Not Affected by FSLLR-NH_2 and LSIGRL-NH_2. One key issue to consider was that the ability of FSY-NH2 and LS-NH_2 to affect the activation of PAR_2 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-NH_2 and LS-NH_2 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-NH_2 or LS-NH_2, was evaluated for its ability to hydrolyze a synthetic peptide substrate, GPNSKGR/SLIGRLTDPYGGC (P20) representing the cleavage/activation sequence of rat PAR_2. Third, in the presence and absence of either FSY-NH_2 or LS-NH_2, the ability of trypsin to cleave and release the N-terminal portion of PAR_2, that is proximal to the receptor cleavage/activation site, was evaluated in KNRK cells expressing rat PAR_2 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 unaltered in the presence of either FSY-NH_2 or LS-NH_2 at the same concentrations (200 μM) that inhibited PAR_2 activation in the cell and tissue assays (Figs. 1–4). Also, FSY-NH_2 and LS-NH_2 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 PAR_2 (GPNSKGR/SLIGRLDP) followed by quenching of the reaction with soya trypsin inhibitor, yielded a proteolysis product (presumably, SLIGRL. . .) capable of activating PAR_2 (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 PAR_2-activating sequence was not affected in the presence of either FSY-NH_2 or LS-NH_2 at a concentration (200 μm) that inhibited trypsin activation of PAR_2 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 PAR_2 activating peptide (no calcium signal, Fig. 6A, third histogram from left; Fig. 6B, top tracing, middle response). Finally, we wanted to investigate

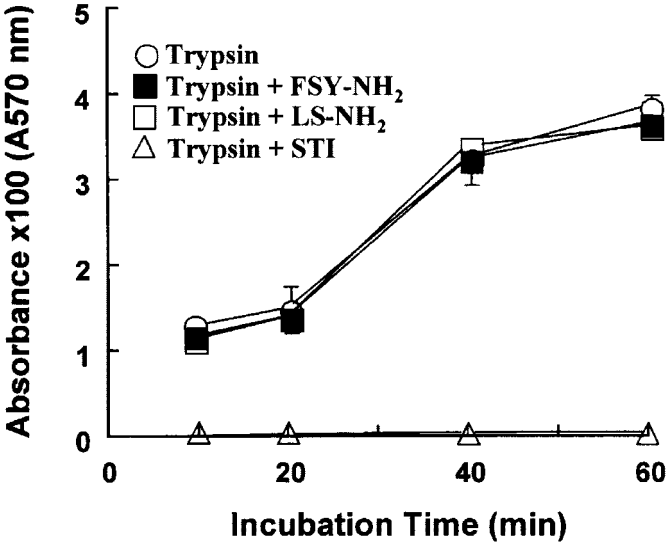

Fig. 5. Lack of effect of FSY-NH_2 or LS-NH_2 on proteolysis of resorufin-labeled casein by trypsin. The time course of proteolysis of the casein substrate by trypsin (increased absorbance at 570 nm: A570) in the absence (○) or presence of FSY-NH_2 (■, 200 μM), LS-NH_2 (□, 200 μM) or soya trypsin inhibitor (△, 1 μg/ml) determined over a 1-h time period at 37°C was monitored as outlined under *Materials and Methods*. Data points represent the averages (± S. E. M, bars) at each time period for triplicate samples.

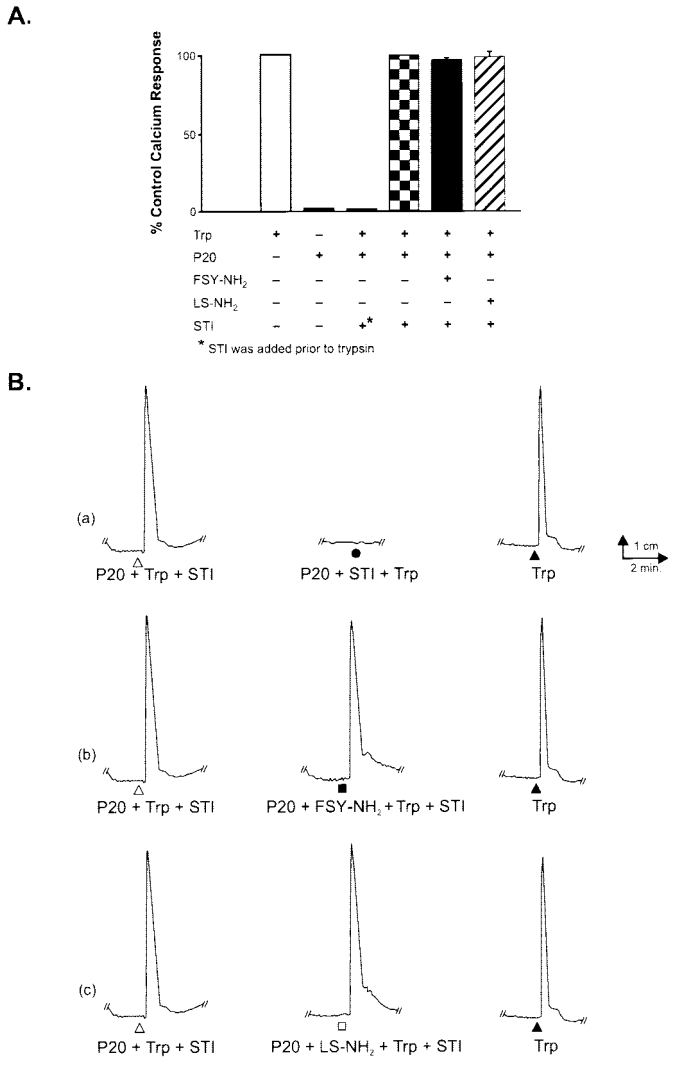

Fig. 6. Proteolytic cleavage by trypsin of a peptide PNSKGR/SLIGRLDTPYGGC (P20) representing the cleavage/activation domain of rat PAR_2 to yield PAR_2-activating peptide: lack of effect of FSY-NH_2 and LS-NH_2 and inhibition by soya trypsin inhibitor. As outlined under *Materials and Methods*, P20 (20 μM) was incubated with 2 nM trypsin at room temperature for 3 min. in the absence or presence of FSY-NH_2 (200 μM), LS-NH_2 (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 PAR_2 KNRK cells, and the calcium signal generated was monitored. The calcium signal caused by the release of PAR_2 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 PAR_2-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 PAR_2-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-NH_2 nor LS-NH_2 affected the trypsin-mediated release of PAR_2-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 PAR_2-KNRK cells by the P20 proteolytic product released by trypsin in the absence or presence of 200 μM added peptide (FSY-NH_2, tracing (b); LS-NH_2, tracing (c)) or STI (1 μg/ml: tracing (a)). The order of addition of P20 peptide, STI, LS-NH_2, FSY-NH_2, and trypsin are shown at the bottom of each tracing. The control responses to trypsin are shown on the right.

whether FSY-NH_2 and LS-NH_2 interfered with the ability of trypsin to release the amino terminal SLAW epitope from PAR_2 by proteolytic activation. As shown in Fig. 7, either in the absence (Fig. 7A, Trp; Fig. 7D) or presence of either FSY-NH_2 (Fig. 7A, FSY-NH_2 + Trp; Fig. 7E) or LS-NH_2 (Fig. 7A, LS-NH_2 +Trp; Fig. 7F), trypsin proteolysis was essentially equally effective in removing the PAR_2 epitope, that is N-terminal to the receptor cleavage/activation site equivalent to P20. The untreated PAR_2 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 PAR_1/PAR_2-related peptides, FSLLRY-NH_2 and LSIGRL-NH_2, while unable on their own to activate PAR_2, were able to block the ability of trypsin to do so, either in a PAR_2 calcium signaling

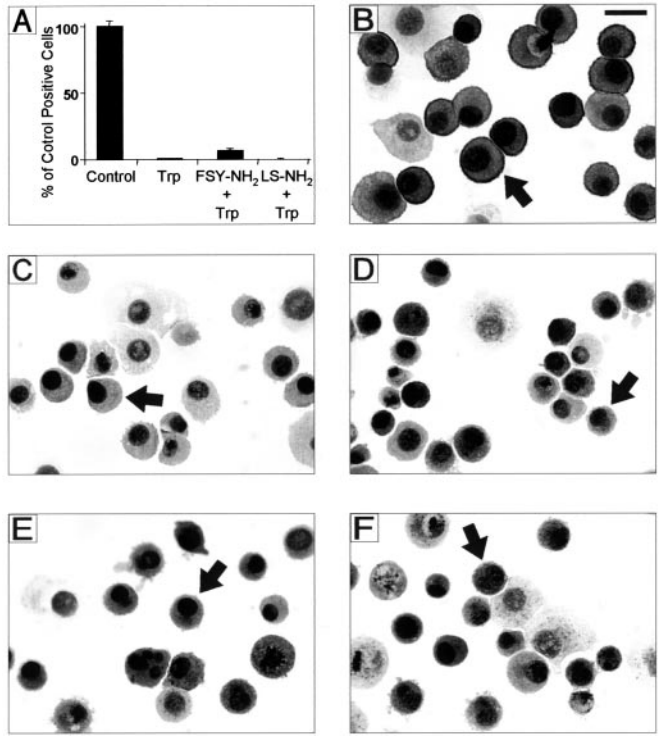

Fig. 7. Cleavage of PAR_2 N-terminal SLAW epitope by trypsin in the presence of FSY-NH_2 and LS-NH_2. As outlined under *Materials and Methods*, permanently transfected PAR_2 expressing KNRK cells were treated with trypsin alone (A, Trp and D), or trypsin in the presence of FSY-NH_2 (A, FSY-NH_2 + Trp and E) or LS-NH_2 (A, LS-NH_2 + 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 PAR_2-expressing KNRK cells (Saifeddine et al., 1998; Al-Ani et al., 1999b) or in intact aorta tissue (Fig. 4) wherein proteolytic activation of PAR_2 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 PAR_2 activation affected receptor triggering by 100 to 400-fold lower concentrations of the PAR_2-activating peptide, SLIGRL-NH_2 (Fig. 1, tracing D and Fig. 4). One possible hypothesis that we considered for the ability of FSY-NH_2 and LS-NH_2 to block trypsin-mediated activation of PAR_2 without affecting activation caused by SL-NH_2 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 PAR_2 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 PAR_2-activating sequence was unaffected, and 3) the ability of trypsin acting on receptor-expressing cells to strip the PAR_2 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 proleolytic activity was responsible for the inhibitory effects of the peptides. This conclusion is supported further by the inability of FSY-NH_2 to inhibit activation of PAR_1 by the serine proteinase thrombin. A working hypothesis we suggest is that although the peptides FSY-NH_2 and LS-NH_2 do not block the ability of trypsin to unmask the tethered receptor-activating ligand sequence, SLIGRL. . . , 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 PAR_2 at which FSY-NH_2 interacts would appear to be receptor-specific, in that the same peptide did not affect the ability of the PAR_1 tethered ligand revealed by thrombin to activate PAR_1. This possible difference between the docking sites of the soluble and tethered ligand in PAR_2 would be in keeping with data obtained for human PAR_1 (Blackhart et al., 2000), demonstrating differences between soluble receptor-activating ligands and the protease-revealed tethered ligand for activating selected PAR_1 receptor mutants. Furthermore, our own work with a mutated PAR_2 receptor having arginines substituted for glutamic acid in extracellular loop 2 showed that the trypsin revealed tethered ligand (SLIGRL. . .) was more effective in activating the receptor than was the comparable soluble receptor-activating ligand, SLIGRL-NH_2 (Al-Ani et al., 1999a). Taken together, the data obtained by us for PAR_2 (Al-Ani et al., 1999a) and for PAR_1 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-NH_2 and LS-NH_2 to block trypsin but not SL-NH_2-mediated activation of PAR_2. 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-NH_2 and LS-NH_2 suggest that any future research for putative PAR_2 receptor antagonists should take both potential mechanisms into account, so as to assess receptor activation both by proteolysis with trypsin and by a receptor-activating peptide such as SL-NH_2.

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References

Al-Ani B, Saifeddine M, and Hollenberg MD (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.

Al-Ani B, Saifeddine M, Kawabata A, and Hollenberg MD (1999a) Proteinase activated receptor 2: role of extracellular of loop 2 for ligand-mediated activation. *Br J Pharmacol* **128:**1105–1113.

Al-Ani B, Saifeddine M, Kawabata A, Renaux B, Mokashi S, and Hollenberg MD (1999b) Proteinase-activated receptor-2 (PAR2): development of a ligand binding assay correlating with activation of PAR2 by PAR1-and PAR2-derived peptide ligands. *J Pharmacol Exp Ther* **290:**753–760.

Bhattacharya A, Smith GF, and Cohen ML (2001) Effect of LY287045, a thrombin/trypsin inhibitor, on thrombin and trypsin-induced aortic contraction and relaxation. *J Pharmacol Exp Ther* **297:**573–581.

Blackhart BD, Emilsson K, Nguyen D, Teng W, Martelli AJ, Nystedt S, Sundelin J, and Scarborough RM (1996) Ligand cross-reactivity within the protease-activated receptor family. *J Biol Chem* **271:**16466–16471.

Blackhart BD, Ruslim-Litrus L, Lu CC, Alves VL, Teng W, Scarborough RM, Reynolds EE, and Oksenberg D (2000) Extracellular mutations of protease-activated receptor-1 result in differential activation by thrombin and thrombin receptor agonist peptide. *Mol Pharmacol* **58:**178–1187.

Bohm SK, Kong W, Bromme D, Smeekens SP, Anderson DC, Connolly A, Kahn M, Nelken NA, Coughlin SR, Payan DG, and Bunnett NW (1996) Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. *Biochem J* **14:**1009–1016.

Compton SJ, Renaux B, Wijesuriya SJ, and Hollenberg MD (2001) Glycosylation and the activation of proteinase-activated receptor 2 (PAR_2) by human mast cell tryptase. *Br J Pharmacol* **134:**705–718.

Dery O, Corvera CU, Steinhoff M, and Bunnett NW (1998) Proteinase-activated receptors: novel mechanisms of signaling by serine proteases. *Am J Physiol* **274:** C1429–C1452.

Hollenberg MD, Saifeddine M, Al-Ani B, and Kawabata A (1997) Proteinase-activated receptors: structural requirement for activity, receptor cross-reactivity and receptor selectivity of receptor-activating peptides. *Can J Phys Pharmacol* **75:**832–841.

Kawabata A, Saifeddine M, Al-Ani B, Leblond L, and Hollenberg MD (1999) Evaluation of proteinase-activated receptor-1 (PAR1) agonists and antagonists using a cultured cell receptor desensitization assay: activation of PAR2 by PAR1-targeted ligands. *J Pharmacol Exp Ther* **288:**358–370.

Kong W, McConalogue K, Khitin LM, Hollenberg MD, Payan DG, Böhm SK, and Bunnett NW (1997) Luminal trypsin may regulate enterocytes through proteinase-activated receptor 2. *Proc Natl Acad Sci USA* **94:**8884–8889.

Kruse HJ, Mayerhofer C, Siess W, and Weber PC (1995) Thrombin receptor-activating peptide sensitizes the human endothelial thrombin receptor. *Am J Physiol* **268:**C36–C44.

Nystedt S, Emilson K, Wahlestadt C, and Sundelin J (1994) Molecular cloning of a potential proteinase activated receptor. *Proc Natl Acad Sci USA* **91:**9208–9212.

Rasmussen UB, Gachet C, Schlesinger Y, Hanau D, Ohlmann P, Van Obberghen-Schilling E, Pouyssegur J, Caszenave JP, and Pavirani A (1993) A peptide ligand of the human thrombin receptor antagonizes alpha-thrombin and partially activates platelets. *J Biol Chem* **268:**14322–14328.

Saifeddine M, Al-Ani B, Cheng CH, Wang L, and Hollenberg MD (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.

Saifeddine M, Al-Ani B, Sandhu S, Wijesuriya SJ, and Hollenberg MD (2001) Contractile actions of proteinase-activated receptor-derived polypeptides in guinea pig gastric and lung parenchymal strips: evidence for distinct receptor systems. *Br J Pharmacol* **132:**556–566.

Saifeddine M, Roy SS, Al-Ani B, Triggle CR, and Hollenberg MD (1998) Endothelium-dependent contractile actions of proteinase-activated receptor-2-activating peptides in human umbilical vein: release of contracting factor via a novel receptor. *Br J Pharmacol* **125:**1445–1454.

Sanger F, Nicklen S, and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74:**5463–5467.

Vergnolle N, Macnaughton WK, Al-Ani B, Saifeddine M, Wallace JL, and Hollenberg MD (1998) Proteinase-activated receptor 2 (PAR2)-activating peptides: identification of a receptor distinct from PAR2 that regulates intestinal transport. *Proc Natl Acad Sci USA* **95:**7766–7771.

Vu T-KH, Hung DT, Wheaton VI, and Coughlin SR (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64:**1057–1068.

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