Proteinase-Activated Receptor 2: Differential Activation of the Receptor by Tethered Ligand and Soluble Peptide Analogs

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ABSTRACT

Activation of rat proteinase-activated receptor 2 (PAR2) by trypsin involves the unmasking of the tethered sequence S37LIGRL42 that either tethered or on its own as a free peptide, activates PAR2. We aimed to determine whether different peptide sequences acting either as trypsin-revealed tethered ligands or as soluble peptides had the same relative activities for triggering the receptor. A comparison was also made between the different soluble and tethered receptor activating sequences in receptor constructs with extracellular loop 2 (ECL2) residues E232E233 (PAR2SR/EE) mutated to R232R233 (PAR2SR/RR); and 3) PAR2 constructs with both the RR mutation in ECL2 and a mutation in the tethered ligand (PAR2SA/RR and PAR2SE/RR); and 2) wild-type PAR2 with ECL2 mutated to R232R233 (PAR2SR/RR); and 3) PAR2 constructs with both the RR mutation in ECL2 and a mutation in the tethered ligand (PAR2SA/RR and PAR2SE/RR); were assessed for receptor-mediated calcium signaling and cell growth inhibition, upon activation either by trypsin or the above-mentioned PAR2APs. Trypsin exerted equivalent and full agonist activity on the PAR2 constructs, causing a maximum response between 20 to 80 nM. In contrast, the PAR2APs as free peptide agonists showed marked potency differences in all wild-type receptors with mutated tethered ligands (SR-NH2 >> SA-NH2 >> SE-NH2) and in all ECL2 RR mutated constructs (SE-NH2 > SR-NH2 > SA-NH2). We conclude that for receptor activation, the trypsin-revealed PAR2 tethered ligand sequence interacts differently for receptor activation than does the same peptide sequence as a free peptide.

PAR2 activation by trypsin involves the proteolytic unmasking of an amino terminal receptor sequence that acts as a tethered ligand (Nystedt et al., 1994). Remarkably, short synthetic peptides, PAR2APs, based on the proteolytically revealed receptor sequence, beginning with serine-37 in the rat receptor, can mimic the action of trypsin in a variety of tissues (Al-Ani et al., 1995; Hollenberg et al., 1997; Cocks et al., 1999). Previously, the amino terminus and ECL2 domain for PAR1 (Gerszten et al., 1994), and the ECL2 domain for PAR2 (Lerner et al., 1996) were found to be directly involved in PAR1 and PAR2 activation by the soluble peptides SFLLRN and SLIGRL, respectively. Replacement of the amino terminal receptor sequence that acts as a tethered ligand (Nystedt et al., 1994) were found to be directly involved in PAR1 and PAR2 activation by the soluble peptides SFLLRN and SLIGRL, respectively. Replacement of the receptor-activating peptides PAR2APs: SLIGRL-NH2 (SR-NH2), SLIGAL-NH2 (SA-NH2), and SLIGEL-NH2 (SE-NH2). Kirsten virus-transformed rat kidney cells stably expressing 1) wild-type PAR2 with site-mutated tethered ligands (PAR2SA/EE and PAR2SE/EE); 2) wild-type PAR2 with ECL2 mutated to R232R233 (PAR2SR/RR); and 3) PAR2 constructs with both the RR mutation in ECL2 and a mutation in the tethered ligand (PAR2SA/RR and PAR2SE/RR); and 4848/1006121

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ABBREVIATIONS: PAR, proteinase-activated receptor; AP, activating peptide; ECL2, extracellular loop 2; SAR, structure-activity relationship; PCR, polymerase chain reaction; KNRK, Kirsten virus-transformed rat kidney; FACS, fluorescence-activated cell sorting; BS, antibody targeted to the cleavage/activation sequence (GPNSKGRSLIGRLDTTP) of rat PAR2; DMEM, Dulbecco’s modified Eagle’s medium; REEC, relative effective concentration.
structure-activity relationship (SAR) studies for short six amino acid receptor-activating peptides derived from the tethered ligand sequences of PAR1 and PAR2 (Natarajan et al., 1995; Hollenberg et al., 1996) have shown the importance of the arginine residue at position 5 of PAR1AP and 2AP, such as SFLLEN-NH₂ and SLIGRL-NH₂. The substitution of an amino acid with an acidic side chain (e.g., glutamic acid) at position 5 of a PAR1AP (e.g., SFLLEN-NH₂) has been found to reduce peptide potency for activating PAR1 by at least 2 orders of magnitude. Similarly, the substitution of an amino acid with a neutral side chain (e.g., alanine) at position 5 of PAR2AP (e.g., SLIGAL) has been found to reduce peptide potency for activating PAR2 by at least 200-fold (Hollenberg et al., 1996). Although a number of structure-activity studies have been done to evaluate the activities of soluble PAR2-activating peptides (Hollenberg et al., 1996, 1997; Maranoff et al., 2001), it is unclear as to whether the activity of these amino acid sequences acting as a tethered ligand would or would not reflect their activities as peptides in solution. We hypothesized that the SARs for the activation of the receptor by the trypsin-revealed tethered ligand sequences may differ from their SARs when acting as soluble peptides. To test this hypothesis, we focused on the PAR2-activating peptide sequences SLIGRL . . . SLIGAL . . . , and SLIGEL . . . . These sequences were selected because, as indicated above, our previous work (Hollenberg et al., 1996, 1997; Al-Ani et al., 1999a) showed that substitution of either alanine or glutamic acid at position 5 of the parent PAR2-activating peptide SLIGRL-NH₂ results in a marked reduction in peptide potency for activating PAR2. Therefore, we prepared and expressed receptor mutants in which the arginine at position 41 of the revealed tethered ligand was changed to either alanine or glutamic acid. When revealed by trypsin, the mutated receptor tethered ligands would then correspond to the synthetic receptor-activating peptides SLIGAL-NH₂ and SLIGEL-NH₂. Compared with the wild-type receptor, designated PAR2SR/EE, these receptors with mutations in the tethered ligand sequence were designated PAR2SE/EE (corresponding to the PAR2AP, SLIGEL-NH₂) and PAR2SA/EE (corresponding to the PAR2AP, SLIGAL-NH₂). Furthermore, we prepared receptors with double mutations, not only in the tethered ligand sequence but also in the acidic tripeptide of ECL2: (PE232E233/PR232R233; Fig. 1). The ECL2 mutations had either the wild-type tethered ligand (PAR2SR/RR) or glutamic acid/alanine mutations in the tethered ligand sequence, designated PAR2SE/RR and PAR2SA/RR. The concentration-effect curves for activation of all receptor constructs by the soluble PAR2APs were compared with the concentration-effect curves for receptor activation by trypsin, which reflected the activity of the tethered ligand sequences.

### Materials and Methods

**PAR2 Cloning and Expression.** Based on the previously determined rat PAR2 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 AB, 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 (Saifeddine et al., 1996).

![Image](image-url)
PAR2SR/EE and PAR2SE/EE, R\textsuperscript{41} was changed to A and E, respectively, and ECL2 amino acids E\textsuperscript{232} and E\textsuperscript{233} were changed to R\textsuperscript{232} and R\textsuperscript{233}. The wild-type PAR2 and PAR2 mutants in pCDNA3 were then transfected into Kirsten virus-transformed rat kidney cells (KNRK; 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\textsuperscript{2} flask; 50–70% confluent). Transfected cells, including wild-type receptor-expressing cells prepared in parallel for this new study, were subcloned in geneticin containing medium (0.6 mg/ml), and receptor-bearing cells were isolated by fluorescence-activated cell sorting (FACS) to yield permanent cell lines with the use of the anti-receptor B5 antibody (Kong et al., 1997; Al-Ani et al., 1999b) generated against a peptide representing the cleavage/activation sequence: GPSKGR/SLLGLRTDPYGCC (\textit{Y}, cleavage site; YGGC added for conjugation). In the cell lines so isolated, >95% of the populations were found to exhibit reactivity with the B5 antibody. In addition, in all mutants the B5 fluorescence intensity on a per cell basis was equivalent. In keeping with our previous work (Al-Ani et al., 1999a), we only maintained and used permanent cell lines that expressed high levels of PAR2 and exhibited equivalent average fluorescence yields on a per cell basis with the B5 antibody. Using the FACS fluorescence signal yielded with the B5 antiserum, the fluorescence intensity of each mutated receptor clone, reflecting cell surface receptor density was expressed (relative fluorescence intensity, R\textsubscript{REL,PAR2SR/EE}; Table 1) relative to the fluorescence yield of the wild-type receptor clone (PAR2SR/EE). In previous work (Al-Ani et al., 1999a), we determined that the wild-type receptor clone expressed about 80,000 cell surface receptor per cell. Cell lines were routinely propagated in genenicin (0.6 mg/ml) containing Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (v/v) fetal calf serum, using 80-cm\textsuperscript{2} plastic T-flasks.

Cells were subcultured by resuspension in calcium-free isotonic saline/EDTA solution, without the use of trypsin.

**Immunocytochemistry.** The wild-type and PAR2 variant cell lines were grown to 90% confluence on eight-chamber Lab-Tek II glass slides (Nalge-Nunc, Naperville IL) in DMEM with geneticin (0.6 mg/ml) and 5% (v/v) fetal calf serum, and fixed in 95% ethanol for 30 min. The cells were washed with phosphate-buffered saline, pH 7.4, between all incubations except after blocking with nonimmune goat serum. After reacting with 3% H\textsubscript{2}O\textsubscript{2} for 10 min, avidin and biotin (Zymed, South San Francisco, CA) for 15 min each and 10% (v/v) nonimmune goat serum (Zymed) for 10 min, the cells were incubated overnight at 4°C with the primary B5 antiserum that detected PAR2 (Al-Ani et al., 1999b) at a dilution of 1:1000. The cells were then incubated with a secondary biotinylated goat anti-rabbit polyclonal antibody (1:100 dilution; Sigma-Aldrich, St. Louis, MO) for 40 min and extravidin-peroxidase (1:100 dilution; Sigma-Aldrich) for 40 min. Finally, the cells were incubated with the 3,3’-diaminobenzidine chromogen (Sigma-Aldrich) at 0.25 μg/ml for 10 min, counterstained with Gill’s #1 hematoxylin (Fisher Scientific, Fair Lawn, NJ), dehydrated through an increasing alcohol gradient and xylene, and mounted in an organic mounting medium (Acrytol; Surgipath, Richmond, IL).

**Measurement of Calcium Signaling Using Fluorescence Emission.** Cells to be used for measurements of the trypsin and peptide-stimulated fluorescence emission (reflecting an increase in intracellular calcium) were grown at 37°C in 80-cm\textsuperscript{2} T-flasks under an atmosphere of 5% CO\textsubscript{2} in room air to about 85% confluence and were disaggregated with calcium-free isosonic phosphate-buffered saline containing 0.2 mM EDTA. Disaggregated cells were pelleted by centrifugation and were resuspended in 1 ml of DMEM/10% fetal calf serum for loading with the intracellular calcium indicator Fluo-3 (Molecular Probes, Eugene, OR) at a final concentration of 22 μM (25 μg ml\textsuperscript{-1}) of Fluo-3-AM ester. Indicator uptake was established over 20 to 25 min at room temperature in the presence of 0.25 mM sulfinpyrazone, after which time cells were washed twice by centrifugation and resuspended with the buffer described below, to remove excess dye. Fluo-3-loaded cells were then resuspended to yield a stock solution (about 6 × 10\textsuperscript{6} cells ml\textsuperscript{-1}) in a buffer of the following composition: 150 mM NaCl, 3 mM KCl, 1.5 mM CaCl\textsubscript{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 fluorescence spectrometer (PerkinElmer Life Sciences, Boston, MA), with an excitation wavelength of 480 nm and an emission recorded at 530 nm. Cell suspension (about 2 ml of approximately 3 × 10\textsuperscript{6} cells ml\textsuperscript{-1}) was maintained in suspension with a stirred (magnetic flea bar) thermostatted cuvette (total volume, 4 ml), and peptide stock solutions were added directly to the suspension to monitor peptide-induced changes in fluorescence. To construct concentration-response curves for fluorescence yield, the signals caused by the addition of test agonists (trypsin or PAR2APs) were expressed as a percentage (%A23187) of the fluorescence peak height yielded by replicate cell suspensions when treated with 2 μM of the ionophore A23187 (Sigma-Aldrich). This concentration of A23187 was at the plateau of its concentration-response curve for a fluorescence response. Previous work (Kawabata et al., 1999; Compton et al., 2000) has shown that the fluorescence response of a cell preparation relative to 2 μM A23187 is a valid reference standard in the determination of concentration-response curves for all PAR agonists. In addition, in previous work we have observed, as expected, that the presence of the extracellular PAR2APs in the cell suspensions does not affect the Fluo-3 signal generated by intracellular calcium indicator, in response to other agonists such as lysophosphatidic acid (Kawabata et al., 1999). Under the assay conditions, the addition of proteinase inhibitors (e.g., amastatin) did not potentiate or diminish the fluorescence response caused by the PAR2APs. Thus, routinely, proteinase inhibitors were not added to the assay cuvettes. Measurements were done using three or more replicate cell suspensions derived from two or more independently grown crops of cells. For calcium transients, figures show exact tracings of the chartrecorder printouts from the fluorescence spectrometer. Values in the histogram and concentration-effect figures represent the average ± S.E.M. (bars).

**Cell Growth Assay.** In six-well (9.6-cm\textsuperscript{2}) culture plates (Nunclon; VWR Canah, Ontario, ON, Canada) KNRK-rPAR2 cells were subcultured without the use of trypsin at 10\textsuperscript{5} cells/well in genenicin (0.6 mg/ml) containing DMEM supplemented with 5% (v/v) fetal calf serum.

<table>
<thead>
<tr>
<th>Construct</th>
<th>EC\textsubscript{50}</th>
<th>R\textsubscript{EC,PAR2SR/EE}</th>
<th>R\textsubscript{FL,PAR2SR/EE}</th>
</tr>
</thead>
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<td>PAR2SR/EE</td>
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<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>PAR2SA/EE</td>
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</tr>
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<td>2.2</td>
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<td>0.8</td>
</tr>
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<td>0.7</td>
</tr>
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<td>1.0</td>
<td>0.9</td>
</tr>
<tr>
<td>PAR2R36A</td>
<td>Inactive</td>
<td>N/A</td>
<td>0.9</td>
</tr>
</tbody>
</table>

N/A, not applicable.
serum and incubated at 37°C for 24 h under an atmosphere of 5% CO2 in room air. Medium was aspirated and cells washed with phosphate-buffered saline before the addition of a low-serum medium (0.2% fetal calf serum) and incubated further for another 24 h. Test agents, trypsin (20 nM), and peptides (50–200 μM) were added in the absence of serum for 1-h incubation at 37°C, before a final concentration of 0.2% fetal calf serum was added. Cells were incubated with the test agents for 48 h, rinsed with phosphate-buffered saline, and harvested for counting, using an improved Neubauer hemacytometer (American Optics, Buffalo, NY). At the low trypsin concentration (20 nM), cells remained attached to the culture dish, as for the peptide-treated cells. Cell cultures showed an increase in cell numbers (ΔN) over the 48-h time period. Inhibition of cell growth was expressed as (1 – (ΔN/ΔNc)) × 100, where ΔN is the increase in the cell number for agonist-treated cells and ΔNc is the increase in cell number for untreated cells.

**Results**

**Expression of PAR2 variants.** A KNRK cell line that lacks the expression of a functional PAR2 (Böhm et al., 1996; Al-Ani et al., 1999b) was used as a recipient cell to express the transfected rat wild-type and mutated PAR2 receptors. Figure 1A shows the substituted amino acids (shaded) in the putative trypsin cleavage/activation and ECL2 sites for the six PAR2 mutants compared with the wild-type receptor. The mutations were targeted to the trypsin cleavage residue (R36), to R41 in the tethered ligand, and to E232 and E233 in the ECL2 domain. The construct PAR2R36A was used as a negative control for receptor activation by the enzyme trypsin, because changing arginine to alanine abolished the trypsin cleavage site. The sketch in Fig. 1B depicts the PAR2 receptor model with the targeted residues for mutation shown (shaded).

The KNRK-PAR2 variants (both wild type and mutants prepared in parallel for this study), isolated by two rounds of purification using the FACS approach, were found to express PAR2 in >95% of the cell population using the B5 anti-receptor antibody that detects an epitope the putative trypsin cleavage/activation site (Kong et al., 1997; Al-Ani et al., 1999b). KNRK-PAR2 variants showed a comparable average mean fluorescence (Fig. 2, A and C; data not shown) using the B5 anti-receptor antibody probe, indicating equivalent receptor expression in all cell lines. Also, relative to the wild-type PAR2 (PAR2SR/EE), the fluorescence intensity yields from FACS in PAR2 mutants (Table 1) were comparable, which indicated an equivalent receptor density among PAR2-expressing cell lines. Equivalent PAR2 expression on the transfected KNRK cells was also visualized with the immunocytochemical detection method using B5 anti-receptor antibody. The cloned cell lines (Fig. 2, B and D; data not shown) had positive and comparable immunocytochemical reactivity to the antibody. In addition, immunoreactivity was not detected either in the “empty” vector-transfected KNRK cell line (Fig. 2E) or in experiments where the B5 antibody was preabsorbed with the receptor-derived peptide immunogen for 2 h before staining the cells. Arrows point to the plasma cell membrane where receptor can be visualized.

**Functional Analysis of PAR2 Variants and Comparison of Agonist Potencies.** Because the seven receptor-bearing cell lines shown schematically in Fig. 1A were observed to have equivalent receptor densities (Fig. 2; Table 1; data not shown), it was possible to determine the relative potency of the PAR2 agonists in each cell line using the calcium assay method, and to study the impact on PAR2 activation of mutations at selected points in the PAR2 receptor tethered ligand sequence, versus receptor activation by comparable soluble peptides having the tethered ligand sequences. Representative calcium signaling responses of PAR2 variants are shown in Fig. 3. Equivalent responses were produced by trypsin in the PAR2 wild type (PAR2SR/EE, Fig. 3A, middle trace) and in all mutant cell lines (Fig. 3, B–D), whereas the magnitude of the responses caused by the soluble peptides differed from each other. In PAR2SR/RR, with the EE232,233RR mutation in ECL2 (Fig. 3B), the trypsin-revealed tethered ligand SLIGRL... fully activated the receptor compared with a minimal activation by the corresponding soluble peptide SLIGRL-NH2 (SR-NH2). Similarly,
NH2 was reduced over 100-fold in PAR2SR/RR. In marked type PAR2SR/EE, the activity of the soluble peptide SLIGRL-tethered ligand sequence (SLIGRL...). Compared with wild the soluble PAR2APs SLIGRL-NH2, SLIGAL-NH2, and SLIGEL-NH2 (SA-NH2, ...) fully activated the receptors compared with the parent wild-type tethered ligand SLIGEL... (Fig. 4, B, D, and F). The scale for time and calcium signal is shown to the right of tracing A. The results are representative of three or more separately conducted experiments with independently grown crops of PAR2-expressing cells.

Concentration-Effect Curves for Trypsin and PAR2APs in PAR2 Variant-Transfected Cell Lines. Concentration-effect curves were obtained for the stimulation of cytosolic calcium release by the action of trypsin and the soluble PAR2APs SLIGRL-NH2, SLIGAL-NH2, and SLIGEL-NH2 in the PAR2 variants (Figs. 4 and 5). In agreement with our previous observations (Al-Ani et al., 1999a) and as shown in Fig. 4A, the soluble peptide SLIGRL-NH2 was a very weak agonist in activating the mutant PAR2 receptor (PAR2SR/RR) that has the ECL2 acidic residues E232E233 (in A), SLIGEL-NH2 (SE-NH2, in C), and SLIGAL-NH2 (SA-NH2, in E) by wild-type PAR2 (PAR2SR/EE) (A), PAR2SR/RR (B), PAR2SE/EE (C), and PAR2SA/EE (D). After challenging cells with the designated agonist, the release of intracellular calcium was monitored by recording fluorescence emission at 530 nm (E530). In each set of tracings, responses to trypsin represent the effects of the revealed tethered ligands, in comparison with responses to the corresponding soluble PAR2APs. Responses were compared relative to the E530 signal yielded in each cell sample by 2 μM of the ionophore A23187 (>). The scale for time and calcium signal is shown to the right of tracing A. The results are representative of three or more separately conducted experiments with independently grown crops of PAR2-expressing cells.

We next examined the impact of simultaneous mutations in two regions of the PAR2 receptor, the amino terminus and ECL2, on the differential activities of the tethered ligands observed that the soluble peptides SLIGEL-NH2 and SLIGAL-NH2 had markedly (more than 100-fold) reduced activity for activating PAR2, we next changed the parent-tethered ligand (SLIGRL...) to SLIGEL... and SLIGAL... and compared the activity of these tethered ligand mutants with corresponding soluble peptides SLIGEL-NH2 and SLIGAL-NH2. There were disparate concentration-effect curves for the soluble PAR2APs but only small shifts in the trypsin concentration-effect curves in the PAR2 wild-type and mutant cells in the calcium signaling assay (Fig. 4, C–F). The activity of SLIGEL-NH2 (Fig. 4C) and SLIGAL-NH2 (Fig. 4E) were markedly reduced in PAR2SR/EE, PAR2SE/EE, and PAR2SA/EE compared with SLIGRL-NH2. In contrast, the corresponding trypsin-revealed tethered ligands SLIGEL... (Fig. 4D) and SLIGAL... (Fig. 4F) were equivalent in activity compared with the parent wild-type tethered ligand SLIGRL... (Fig. 4B, D, and F).
versus soluble peptides (Fig. 5). The concentration-effect curves for trypsin and PAR2APs were compared for ECL2 mutant (PAR2SR/RR) versus mutants in which both ECL2 and the tethered ligand were changed (PAR2SE/RR and PAR2SA/RR). Different magnitudes of calcium responses were produced by the soluble free peptides SLIGRL-NH$_2$ (Fig. 5, A and C), SLIGEL-NH$_2$ (Fig. 5A), and SLIGAL-NH$_2$ (Fig. 5C) in the PAR2 variants. It was of particular note that SLIGAL-NH$_2$ exhibited minimal activity in the EE232,233RR mutants (Fig. 5C, right-hand curves) but caused efficient activation of the PAR2SA/RR receptor as a tethered ligand (Fig. 5D). In comparison with the soluble peptides, the corresponding trypsin-revealed tethered ligands SLIGRL... (Fig. 5, B and D), SLIGEL... (Fig. 5B), and SLIGAL... (Fig. 5D) were about equally effective and fully activated their receptors when unmasked by trypsin. Furthermore, in both constructs (PAR2SA/RR and PAR2SR/RR), SLIGAL-NH$_2$ (Fig. 5C) behaved as a very weak agonist (up to 800 $\mu$M) compared with the action of SLIGRL-NH$_2$ (Fig. 5A).

Relative Activities (R$\text{EC}$ values) of Trypsin and PAR2APs for Activating PAR2 Variants. The activities of trypsin and the soluble peptide agonists in the mutated receptor constructs (calcium response) were calculated relative to their activities in the wild-type PAR2, as we have done previously for PAR2 receptor mutants (Al-Ani et al., 1999a) (R$\text{EC}_{\text{PAR2/SR/EE}}$; Tables 1 and 2). Use of the R$\text{EC}$ values permits potency comparisons between agonist concentration-effect curves without requiring a precise estimate of $E_{\text{max}}$ and EC$_{50}$ values. As an alternative way of expressing the relative activities of the soluble peptides SLIGRL-NH$_2$ and SLIGEL-NH$_2$, their potencies in each cell line were expressed relative to the action of SLIGRL-NH$_2$, which represents the wild-type soluble PAR2AP (R$\text{EC}_{\text{SLIGRL-NH2}}$; Table 3). Half-maximum effective concentration (EC$_{50}$) values for trypsin in the different PAR2 constructs were also determined from trypsin concentration-effect curves, wherein it was possible to estimate $E_{\text{max}}$ (Table 1).

The potency of trypsin for activating the PAR2 variants is summarized in Table 1. With the exception of the negative control that was resistant to trypsin activation (PAR2R36A; data not shown), trypsin showed a comparable potency for activating all of the PAR2 variants. The EC$_{50}$ values for trypsin ranged from 1.5 to 4 nM. The activity of trypsin in the mutant receptor cell lines, relative to its activity in the PAR2-wild type cell line was equivalent, using either the R$\text{EC}$ values obtained from the parallel portion of the concentration-response curves or the estimated EC$_{50}$ values (Table 1).

In contrast, the relative potencies of the PAR2APs in the receptor mutants varied considerably, as shown in Table 2. Relative to the wild-type PAR2 receptor (PAR2SR/EE), the activity of the soluble peptide SLIGRL-NH$_2$ dropped markedly in the ECL2 EE232,233RR mutants (PAR2SR/RR, 112-fold; PAR2SA/RR, 54-fold; and PAR2SE/RR, 45-fold). In contrast, there was only a slight decline (about 2-fold) in SLIGRL-NH$_2$ activity in the receptors with mutations in the tethered ligand (PAR2SA/EE; PAR2SE/EE) compared with PAR2SR/EE.

**Table 2**

Relative receptor activities for the soluble peptide agonists SLIGRL-NH$_2$, SLIGAL-NH$_2$, and SLIGEL-NH$_2$ compared with the activities in the wild-type receptor PAR2SR/EE.

<table>
<thead>
<tr>
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<th>SLIGAL-NH$_2$</th>
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<td>PAR2SR/RR</td>
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**Table 3**

SLIGAL-NH$_2$ and SLIGEL-NH$_2$ activities relative to SLIGRL-NH$_2$ in different receptor constructs.

Different PAR2 receptors were expressed in KNRK cells and the calcium signal in response to increasing concentrations of the soluble peptides was used to determine the concentration-response curves. The activities of SLIGAL-NH$_2$ and SLIGEL-NH$_2$ in all cell lines were expressed relative to that of SLIGRL-NH$_2$ (R$\text{EC}_{\text{SLIGRL-NH2}} = 1$). In each cell line, the concentration of SLIGAL-NH$_2$ or SLIGEL-NH$_2$ for a given response in the linear portion of its concentration-effect curve was divided by the concentration of SLIGRL-NH$_2$ causing the same calcium response. Average R$\text{EC}$ values (for which S.E.M. values were 10% or less) were obtained from three or more points along the parallel portions of the concentration-effect curves (Figs. 4 and 5). R$\text{EC}$ values greater than 1.0 designate an agonist that is less potent than the parent PAR2AP SLIGRL-NH$_2$.

<table>
<thead>
<tr>
<th>Construct</th>
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</tbody>
</table>
the wild-type receptor PAR2SR/EE. The pattern of relative activation by SLIGAL-NH₂ was similar to that of SLIGRL-NH₂, in that the activity of SLIGAL-NH₂ was reduced much more in the ECL2 EE232,233RR mutants (PAR2SR/RR, 30-fold; PAR2SA/RR, 22-fold; and PAR2SE/RR, 12-fold) than in receptors with mutations only in the tethered ligand (PAR2SA/EE, 1.7-fold; and PAR2SE/EE, 1.7-fold relative to the activity of SLIGRL-NH₂ in the wild-type receptor). On the other hand, relative to the receptors with the wild-type ECL2 motif E₂₃₂E₂₃₃, the potency of SLIGEL-NH₂ was increased (2–5-fold) in all of the ECL2 RR mutants (Tables 2 and 3, right-hand column; reduced R_EC values).

As shown in Table 3, the relative activities of the soluble PAR2AP analogs SLIGEL-NH₂ and SLIGAL-NH₂ were calculated in each individual cell line relative to the action of the parent PAR2AP SLIGRL-NH₂ (R_EC, SLIGRL-NH₂). The data show marked differences in potencies among the soluble receptor-activating peptides. For instance, in all receptors with the EE232,233RR mutation in ECL2, SLIGEL-NH₂ was either slightly more potent or of equivalent potency compared with SLIGRL-NH₂, whereas in all of the receptors with the wild-type EE sequence in ECL2, SLIGEL-NH₂ was 80- to 130-fold less potent than SLIGRL-NH₂. In contrast, SLIGAL-NH₂ was 10- to 30-fold less potent relative to SLIGRL-NH₂ in all of the receptors studied (Table 3, middle column). In summary, the potency orders for all PAR2 agonists in the six cell lines as shown in Figs. 3 to 5, and summarized in Tables 1 to 3 were 1) for receptors with wild-type ECL2, but with a mutated tethered ligand: trypsin ≫ SLIGRL-NH₂ ≫ SLIGAL-NH₂ ≫ SLIGEL-NH₂; and 2) for all ECL2 EE232,233RR mutated constructs: trypsin ≫ SLIGEL-NH₂ > SLIGAL-NH₂ > SLIGRL-NH₂ > SLIGEL-NH₂. Notwithstanding, as already pointed out the potency of trypsin for receptor activation via the revealed tethered ligand was equivalent among all of the receptor variants, including the wild-type receptor (Table 1).

**Cell Growth Inhibition.** We also wanted to investigate whether the observed differences in the potencies for receptor activation by trypsin and the soluble peptides as monitored in the rapid calcium response might also be reflected by differences in potencies for causing a delayed cellular response (inhibition of cell growth). However, we do recognize the complexity of monitoring a delayed cellular response, in this case, inhibition of cell growth, in response to PAR2 activation. This complexity arises because of a lack of knowledge about intracellular signals that regulate growth inhibition and because of a possible interaction of other unknown factors in this process. In our study, we compared the cell growth inhibition obtained from activation of the PAR2SR/RR and wild-type PAR2SR/EE constructs having the same revealed tethered ligand sequences but a difference in ECL2 motif. Cell growth inhibition was monitored in KNRK cells transfected with the rat PAR2 variants after preliminary work with the ECL2 receptor mutant PAR2SR/RR (Al-Ani et al., 1999a). In keeping with our previous work, the data in Fig. 4, A and B, illustrate the proximity of the concentration-response curves for trypsin in PAR2SR/EE and PAR2SR/RR, but the marked separation between distinct concentration-effect curves for the soluble PAR2AP SLIGRL-

![Fig. 6. Histograms representing the inhibition of cell growth in wild-type PAR2 PAR2SR/EE, and mutant PAR2 PAR2SR/RR-transfected KNRK cells. Subconfluent cells were starved for 24 h in 0.2% fetal calf serum before PAR2 agonists were added and incubated for additional 48 h and then cells were harvested and counted. Cell growth inhibition (% = (1 – (A_Nt/A_Nc) × 100, where A_Nt represents the increase in the number of untreated “control” cells. The effect of the reverse peptide LSIGRL-NH₂ (200 μM) on cell growth is also shown. Values represent the averages (± S.E.M., bars) of three or four separate experiments in which measurements were made using three replicates.](Image)

**Discussion**

A principal objective of our study was to determine whether PAR2 peptide sequences acting either as trypsin-revealed tethered ligands or as free soluble peptides had the same relative activities for triggering the receptor. Therefore, structure-activity relationship studies were done both for mutated receptors, with changes in the trypsin-revealed tethered ligand sequence and with soluble receptor-activating peptides having a sequence identical to the first six amino acids of the trypsin-revealed tethered ligand. The activities of these sequences either as tethered ligands or as soluble peptides were evaluated in receptors having either wild-type extracellular loop 2 or a mutated ECL2 sequence (EE232,233RR) at a site thought to play a major role in receptor activation by soluble peptides (Lerner et al., 1996; Al-Ani et al., 1999a). The relative activities of the tethered ligand sequences were reflected by the relative potencies of trypsin for receptor activation.

One of our main findings was that there were very small differences in the potency (EC₅₀ values) or maximum effect for trypsin, working via the revealed tethered ligand, to activate a number of PAR2 variants with mutations: 1) in the tethered ligand sequence (position 5 of the trypsin-exposed peptide), 2) in extracellular loop 2 (EE232,233RR) or 3) concurrently at sites both in the tethered ligand and in ECL2. The data obtained with trypsin therefore indicated that, irrespective of the mutated receptor sequences studied, full receptor activation was achieved. These results, demonstrating comparable potencies and effects for trypsin in the several receptor mutants, were entirely in accord with our preliminary work with the ECL2 receptor mutant PAR2SR/RR (Al-Ani et al., 1999a). In keeping with our previous work, the data in Fig. 4, A and B, illustrate the proximity of the concentration-response curves for trypsin in PAR2SR/EE and PAR2SR/RR, but the marked separation between distinct concentration-effect curves for the soluble PAR2AP SLIGRL-
NH₂ in the same two receptor constructs. From the data obtained for activation of the receptors with trypsin, one can conclude that the trypsin-revealed tethered ligand sequences SLIGRL. . ., SLIGAL. . ., and SLIGEL. . . display equivalent abilities to activate the receptor, whether or not ECL2 possesses the EE232,233RR acidic to basic mutation.

In contrast with the comparable EC₅₀ values for trypsin in the various receptor mutants, the relative potencies of the soluble PAR2APs SLIGRL-NH₂, SLIGAL-NH₂, and SLIGEL-NH₂ differed considerably. These EC₅₀ differences were evident not only in the wild-type receptor (PAR2SR/EE; Table 1), in keeping with our previous findings in intact tissue and receptor-expressing cells (Hollenberg et al., 1996; Al-Ani et al., 1999a), but also in receptors with the EE232,233RR mutation in ECL2 (Tables 2 and 3). For instance, in all receptors with the wild-type ECL2 (E²²³E²³³), either SLIGAL-NH₂ or SLIGEL-NH₂ as free peptides were 20- to 130-fold less potent than the tethered ligand-derived peptide SLIGRL-NH₂. These large differences can be compared with the relatively small (2-fold at most) differences in the EC₅₀ values for trypsin in receptors possessing the corresponding mutated tethered ligand sequences (see PAR2SA/EE and PAR2SE/EE; Table 1). In the receptors with the mutation in ECL2 (EE232,233RR), SLIGAL-NH₂ was about 10-fold less potent than SLIGRL-NH₂ (Table 3), whereas the comparable tethered ligand SLIGEL. . . revealed by trypsin was only 2- to 3-fold less active than the parent sequence SLIGRL. . . (Table 1). In line with our previous observations (Al-Ani et al., 1999a), and in keeping with E²⁶⁰ mutations in human PAR1 (Nanevicz et al., 1995), SLIGEL-NH₂ relative to SLIGRL-NH₂ was somewhat more active in all of the EE232,233RR mutants (Table 3) than it was in those receptors having the wild-type ECL2 sequence (E²²³E²³³). In comparing the relative activities (Rₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑₑ电厂) that the ECL2 acidic to basic mutation (Rₑₑₑₑₑₑₑₑₑₑₑₑₑ电厂) of SLIGEL-NH₂ be-

increase the free peptide. Whether this increase is due to differential ligand-receptor binding, receptor-G protein coupling and signaling, or both remains to be determined. It is also feasible that the intact N-terminal sequence might partially hinder access of the peptide to its binding site. However, the lack of shift of the concentration-effect curve for trypsin in the SR/EE and SR/RR receptors (Fig. 4B) would argue against a dramatic change in receptor conformation that might be responsible for the marked difference in potency of SLIGRL-NH₂ in the two PAR2 receptors (Fig. 4A).

Observations with the soluble PAR-APs SFLLRN-NH₂ and SLIGEL-NH₂ in human PAR1 (Nanevicz et al., 1995) and in rat PAR2 (Al-Ani et al., 1999a), respectively, using receptors with complementary acidic-to-basic changes in ECL2 (human PAR1, E260R; rat PAR2, EE232,233RR) indicate that there may be charge complementation between the basic arginine residue at position 5 of the soluble PAR-APs, and acidic glutamic acid receptor residues in ECL2 (E²⁶⁰ for human PAR1; E²²³E²³³ in rat PAR2). It was suggested (Nanev-

icz et al., 1995; Al-Ani et al., 1999a) that the ECL2 acidic motif (E²⁶⁰ in human PAR1 or E²²³E²³³ in rat PAR2) might play an important role in interacting with the basic arginine residue in the proteinase revealed tethered ligand (SFLLRN. . . for human PAR1; SLIGRL. . . for rat PAR2). Our new data, although supporting that hypothesis for the action of soluble PAR-APs, argue strongly against such a charge complementation for the proteinase-revealed tethered ligand sequence. The strongest data arguing against charge complementation between position 5 of the revealed tethered PAR2 ligand and the charge(s) at position(s) E²²³E²³³ of ECL2 can be seen in Figs. 4, B and F, 5D, and 6. Clearly, having either the same or complementary side chains charges at positions 5 of the tethered ligand and positions 232/233 in ECL2 of PAR2 had little impact on the activation of the receptor by the trypsin-revealed tethered ligand. This conclusion was supported by data obtained both with the calcium signaling assay (Figs. 4 and 5) and with the growth inhibition assay, where trypsin was equally effective in ei-

ther the wild-type receptor or the PAR2SR/RR mutant (Fig. 6). Conversely, as mentioned above, such charge changes in ECL2 of the PAR1 and PAR2 receptors have been observed to have a marked influence on the activities of the soluble PARAPs with differing charges at position 5. Our data complement well and considerably extend work done with human PAR1 (Blackhart et al., 2000). In that study, which appeared upon completion of our work, the change of an acidic to neutral mutation in ECL2 (E260A) had a very modest effect on activation by thrombin and essentially no effect on activation by the soluble PAR1AP SFLLRN-NH₂ (Nanevicz et al., 1995). Indeed, other data obtained with PAR1 mutants imply a much more prominent role for the aspartic acid residue at position 256 and the glutamic acid residue at position 347 of human PAR1 for the activity of soluble PAR1APs compared with E²⁶⁰ (Blackhart et al., 2000). Be-

cause our data for trypsin activation of the PAR2 variants compared with equivalent activation by thrombin (Blackhart et al., 2000). For instance, in human PAR1 receptor mutants either missing a portion of extracel-

lular loop 3 (hPAR1: ΔE339–344) or with an aspartic acid to alanine mutation at position 256 of ECL2 (hPAR1 D256A), the soluble PAR1AP SFLLRN-P-NH₂ that still bound to the mutated receptor lacked activity, whereas the same sequence revealed by thrombin as a tethered ligand seemed active (Blackhart et al., 2000). The work with the PAR1 mutants did not, however, explore in any way the structure-activity
relationships for the tethered ligand sequence. Taken together, our new work with PAR2 variants with alterations in the tethered ligand sequence and the parallel work with PAR1 receptors resistant to activation by a soluble PAR1AP, but activated by thrombin, indicate that the SARs for the receptor-triggering sequence acting as a tethered ligand seem to differ from the SARs for the soluble receptor-activating peptides. Thus, further work remains to be done to identify with confidence, the extracellular receptor docking site(s) of the revealed tethered ligands for both PAR1 and PAR2.

References


