Identification and Characterization of Novel Small-Molecule Protease-Activated Receptor 2 Agonists


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ABSTRACT

We report the first small-molecule protease-activated receptor (PAR) 2 agonists, AC-55541 [N-[1-[3-bromo-phenyl]-eth-(E)-ylidene-hydrazinocarboxyl][4-oxo-3,4-dihydro-phthalazin-1-yl]-methyl]-benzamide] and AC-264613 [2-oxo-4-phenyl-pyrrrolidine-3-carboxylic acid [1-(3-bromo-phenyl)-(E/Z)-ethylidene]-hydrazide], each representing a distinct chemical series. AC-55541 and AC-264613 each activated PAR2 signaling in cellular proliferation assays, phosphatidylinositol hydrolysis assays, and Ca2+ mobilization assays, with potencies ranging from 200 to 1000 nM for AC-55541 and 30 to 100 nM for AC-264613. In comparison, the PAR2-activating peptide 2-furoyl-LIGRLO-NH2 had similar potency, whereas SLIGRL-ethylidene-hydrazide had no significant affinity for PAR2 receptors. Neither AC-55541 nor AC-264613 had activity at any of the other PAR receptor subtypes, nor did they have any significant affinity for over 30 other molecular targets involved in nociception. Visualization of EYFP-tagged PAR2 receptors showed that each compound stimulated internalization of PAR2 receptors. AC-55541 and AC-264613 were well absorbed when administered intraperitoneally to rats, each reaching micromolar peak plasma concentrations. AC-55541 and AC-264613 were each stable to metabolism by liver microsomes and maintained sustained exposure in rats, with elimination half-lives of 6.1 and 2.5 h, respectively. Intrapaw administration of AC-55541 or AC-264613 elicited robust and persistent thermal hyperalgesia and edema. Coadministration of either a tachykinin 1 (neurokinin 1) receptor antagonist or a transient receptor potential vanilloid (TRPV) 1 antagonist completely blocked these effects. Systemic administration of either AC-55541 or AC-264613 produced a similar degree of hyperalgesia as was observed when the compounds were administered locally. These compounds represent novel small-molecule PAR2 agonists that will be useful in probing the physiological functions of PAR2 receptors.

Protease activated receptors (PARs) are a family of four G-protein-coupled receptors (PAR1, PAR2, PAR3, and PAR4) that are self-activated by tethered ligands exposed by proteolytic cleavage of the extracellular amino terminus. PAR1, PAR3, and PAR4 are activated by trypsin, whereas PAR2 and, to a lesser degree, PAR4, are activated by trypsin. In addition, PAR2 can be proteolytically activated by a variety of other substances including trypsin, factor Xa/tissue factor/factor VIIa, and the dust mite allergens Der p3 and Der p9, among others. Exposure to soluble synthetic peptides mimicking the effects of activating proteases (for review, see Ramachandran and Hollenberg, 2008).

PAR2 receptors have been implicated in numerous physiological processes necessitating therapeutic intervention, especially pain and inflammation (Vergnolle et al., 1999, 2001; Steinhoff et al., 2000) and syndromes with a strong inflammatory component, including colitis (Fiorucci et al., 2001; Nguyen et al., 2003; Hansen et al., 2005; Cenac et al., 2007), gastritis (Kawabata et al., 2005), pancreatitis (Kawabata et al., 2006), asthma and pulmonary disease (Cocks et al., 1999;
Ricciardolo et al., 2000; Cicala et al., 2001; Moffatt et al., 2002; Schmidlin et al., 2002; Kawabata et al., 2004b; De Campo and Henry, 2005; Ebeling et al., 2005; Morello et al., 2005; Su et al., 2005), and arthritis (Ferrell et al., 2003; Kelso et al., 2006). PAR2 receptors are widely distributed, with expression throughout the central nervous system, gastrointestinal system, pulmonary system, liver, kidney, exocrine glands (pancreas, salivary glands, tear ducts), heart, vascular tissue, reproductive organs, skin and inflammatory cells including eosinophils, mast cells, macrophages, and neutrophils (D’Andrea et al., 1998; for review, also see Henry, 2006; Ramachandran and Hollenberg, 2008). PAR2 receptors are found on primary spinal afferent neurons coexpressed with substance P, calciotinin gene-related peptide (CGRP), and transient receptor potential vanilloid receptor (TRPV) 1 (Steinhoff et al., 2000; Amadesi et al., 2004; Dai et al., 2004). Convincing evidence has been reported showing that PAR2 receptors mediate many of their actions through a neurogenic mechanism (Ricciardolo et al., 2000; Steinhoff et al., 2000; Fiorucci et al., 2001; Vergnolle et al., 2001; Nguyen et al., 2003; Amadesi et al., 2004; Kawabata et al., 2005; Su et al., 2005; Cenac et al., 2007). Given these physiological roles for PAR2 receptors, PAR2 agonists would be expected to exacerbate most nociceptive and inflammatory processes; however, PAR2 also exerts a protective effect in many settings (Cocks et al., 1999; Cicala et al., 2001; Fiorucci et al., 2001; Kawabata et al., 2004b, 2006; De Campo and Henry, 2005; Morello et al., 2005; D’Agostino et al., 2007), especially in models of asthma and pulmonary inflammation; thus, there may be therapeutic roles for PAR2 agonists (De Campo and Henry, 2006; Henry, 2006). The therapeutic potential for PAR2 antagonists, which relieve symptoms in models of rheumatoid arthritis, appears clearer (Kelso et al., 2006; Cenac et al., 2007).

Little progress has been made in identifying nonpeptidic, nonproteolytic agonists or antagonists of PAR2 receptors. Ideally, such compounds would be metabolically stable, small organic compounds. Mainly peptides and peptide mimetics have been identified as PAR2 ligands, including the PAR2 APs SLIGKV, SLIGRL and their amidated derivatives, and trans-cinnamoyl-LIGRLO-NH₂ (Ramachandran and Hollenberg, 2008). In general, the low potency and susceptibility to proteolytic degradation of these compounds are major limitations, particularly for in vivo use. Recently, peptide agonists of PAR2, such as 2-furyl-LIGRLO-NH₂, with significantly enhanced potency and stability have been developed (Ferrell et al., 2003; Kawabata et al., 2004a; McGuire et al., 2004), PAR2 peptide antagonists have been reported; however, their mechanism of action is unclear because they do not block the actions of PAR2 APs and they do not inactivate trypsin (Al-Ani et al., 2002). Recently, a low-potency small-molecule antagonist for PAR2 has been described (Kelso et al., 2006).

Utilizing high-throughput functional screening, we have identified the first nonpeptide agonists at the PAR2 receptor. We have characterized these compounds in a variety of in vitro and in vivo functional assays. These compounds are highly selective for PAR2 over the other PAR subtypes, are metabolically stable, and have persistent activity in vivo. These compounds will be useful in further exploring the physiological roles of PAR2 receptors.

Materials and Methods

Ligands. SLIGRL-NH₂, SFLLRN-NH₂, AYPGKF-NH₂, and 2-furyl-LIGRLO-NH₂ were obtained from Peptides International Inc. (Louisville, KY). AC-55541 and AC-264613 were synthesized at ACADIA (see Seitzberg et al., 2008). Compound structure was verified by NMR. Puricy was greater than 99%, measured by high-performance liquid chromatography and gas chromatography.

Cell Culture. NIH-3T3 cells (CRL 1658; American Type Culture Collection, Manassas, VA) were incubated at 37°C in a humidified atmosphere (5% CO₂) in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 25 mM glucose, 4 mM L-glutamine, 50 U/ ml penicillin G, 50 U/ml streptomycin (Invitrogen), and 10% fetal calf serum (Sigma-Aldrich, St. Louis, MO) or 25% Ultraculture synthetic supplement (Lonza Walkersville, Inc., Walkersville, MD). Human embryonic kidney (HEK) 293T (CCL-11268; American Type Culture Collection) cells were cultured similarly, except that 10% calf serum was substituted for 10% fetal calf serum.

Constructs. The human PAR1, PAR2, PAR3, and PAR4 receptors used in this study were cloned by polymerase chain reaction using oligonucleotides derived from the GenBank accession entries M62424, U34038, U92971, and AF055917, respectively, and subcloned into pS1 (Promega, Madison, WI) expression vectors. Polymerase chain reaction reactions were performed using Pfu Turbo (Stratagene, La Jolla, CA). All mutations described were generated using the Quickchange protocol (Stratagene). PAR2-S37P-EYFP constructs in pS1 expression vectors were produced by ligating polymerase chain reaction products of PAR2 or PAR2-S37P with the stop codons removed in frame with EYFP fused to the carboxy terminus. All clones were sequence-verified.

Functional Assays. Receptor selection and amplification technology (R-SAT) assays were performed as described previously (Burstein et al., 2006), with the following modifications. In brief, cells were plated 1 day before transfection using 7 × 10⁵ cells in 0.1 ml of media per well of a 96-well plate (Falcon; BD Biosciences Discovery Labware, Bedford, MA). Cells were transiently transfected with 10 ng of receptor DNA and 30 ng of pS1-β-galactosidase (Promega) per well of a 96-well plate using Polyfect (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. One day after transfection, medium was changed, and cells were combined with ligands in DMEM supplemented with 25% Ultraculture synthetic supplement (Lonza Walkersville, Inc.) instead of calf serum to a final volume of 200 μl/well. After 5 days in culture, β-galactosidase levels were measured essentially as described (Burstein et al., 2006). Cells were rinsed with phosphate-buffered saline (PBS), pH 7.4, before the addition of 200 μl of PBS supplemented with 3.5 mM O-nitrophenyl-β-d-galactopyranoside and 0.5% Nonidet P-40 (both Sigma-Aldrich). After incubation (2–4 h), the plates were read at 420 nm on a plate reader (BioTek EL 310; BioTek Instruments, Winooski, VT; or Molecular Devices, Sunnyvale, CA).

Phosphatidylinositol Hydrolysis Assays. Phosphatidylinositol (PI) hydrolysis assays were performed as follows. For PAR2 WT, HEK 293T cells were seeded at 10,000 cells/well in DMEM (Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 37°C humidified atmosphere containing 5% CO₂. Eighteen hours later, the cells were transfected as described above with the indicated plasmid DNAs (30 ng/well of a 96-well plate). Approximately 20 to 24 h after transfection, the cells were washed and labeled overnight with DMEM culture medium containing 0.2 μCi of newborn calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 37°C humidified atmosphere containing 5% CO₂. Eighteen hours later, the cells were transfected as described above with the indicated plasmid DNAs (30 ng/well of a 96-well plate). Approximately 20 to 24 h after transfection, the cells were washed and labeled overnight with DMEM culture medium containing 0.2 μCi of NET1114 (37 MBq/ml; PerkinElmer Life and Analytical Sciences, Waltham, MA) per well (0.1 ml). The cells were washed and incubated with Hanks’ balanced salt solution (Invitrogen) supplemented with 1 mM CaCl₂, 1 mM MgCl₂, 10 mM LiCl, and 0.2% bovine serum albumin for 45 min. The buffer was removed, and the cells were incubated for another 45 min at 37°C in the same buffer with the concentrations of freshly made ligands indicated in the figure legends. The reaction was stopped by exchange with ice-
cold 20 mM formic acid, and the total [3H]inositol phosphate (IP1, IP2, and IP3) formation was determined by ion-exchange chromatography on 1-ml minicolumns loaded with 200 μl of a 50% suspension of AG 1-×8 resin (200–400 mesh, formate form; Bio-Rad, Hercules, CA). The columns were washed with 1 ml of 40 mM ammonium hydroxide, pH 9, after loading the cell extracts and then eluted with 0.4 M of 2 M ammonium formate in 0.1 M formic acid. The eluates (0.1 ml) were loaded on LumaPlate-96 plates (Yttrium silicate scintillator coated; PerkinElmer Life and Analytical Sciences), air-dried overnight, and counted on a Microplate Scintillation and Luminescence Counter (TopCount NXT; PerkinElmer Life and Analytical Sciences). For PAR2 S37P, PAR1 S42P, and PAR R47A, HEK 293T cells were seeded at 4.2 × 10^4 cells per 10-cm dish as described above. Eighteen hours later, the cells were transfected using FUGENE-HD (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions with the indicated plasmid DNAs (10 μg/plate). Approximately 20 to 24 h after transfection, the cells were lifted with PBS/EDTA and resuspended in isoinositol-free DEMEM (Caisson Laboratories, North Logan, UT) (100-μl total volume/well) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mg/ml), and 2 μg/ml myoinositol (37 MBq/ml NETI1114; PerkinElmer Life and Analytical Sciences) and seeded in a 96-well plate at 20,000 cells/well. Twenty-four hours later, medium was carefully removed, and the cells were rinsed with 2 × 200 μl/well serum-free medium containing 0.3% BSA (wash medium). Wash medium (50 μl) containing 10 mM LiCl was added per well and incubated at 37°C for 15 min, followed by the addition of another 50-μl wash medium containing 10 mM LiCl and 2× concentrations of the indicated ligands and incubation for 1 h. The assay was terminated by carefully removing the medium and lysing the cells with 50 μl of 0.1 M formic acid for 20 min at room temperature. Diluted SPA beads (80 μl; GE Healthcare, Chalfont St. Giles, UK), followed by 30 μl of each cell lysate, was added per well of PerkinElmer Life and Analytical Sciences Pico plates, and the plates were sealed and placed on a shaker for at least 1 h. Radioactivity was determined using a TopCount.

Ca²⁺ Mobilization Assays. Intracellular changes in calcium concentrations due to activation of PAR2 receptors were detected using the calcium binding bioluminescence protein aequorin, which was expressed as part of a tripartite chimeric protein, MT-GFP-AEQ, as described previously (Burstein et al., 2006), with the exception that KNRK cells (CRL-1569 from American Type Culture Collection) transiently transfected with human PAR2 receptors using the Geneporter II transfection kit (Genlantis, San Diego, CA) were used for these studies.

Data Analysis. Concentration-response graphs for all functional assays were plotted, and EC₅₀ values were determined by nonlinear regression analysis using Prism software (GraphPad version 4.0; GraphPad Software Inc., San Diego, CA) according to the following equation:

\[ Y = \text{Bottom} + \left( \frac{\text{Top} - \text{Bottom}}{1 + 10^{(\text{LogEC}_{50} - X)}} \right) \]  

where X is the logarithm of concentration, Y is the response, Y starts at the bottom and goes to the top with a sigmoid shape. Allowing the Hill coefficient to vary did not significantly change the fits of the curves; thus, the Hill coefficient was constrained to unity.

Immunofluorescence Confocal Microscopy. Transiently transfected HEK 293T cells expressing PAR2-EYFP or PAR2-S37P-EYFP receptors were grown overnight on CC2 chamber slides (Nalge Nunc International, Rochester, NY). Treatments with 100 μM SLIGRL-NH₂, 1 μM 2-furoyl-LIGRLO-NH₂, 3 μM 55541, and 1 μM 264613 were carried out at 37°C for 15, 30, or 60 min. After treatment, cells were fixed for 10 to 15 min at room temperature with 3.7% paraformaldehyde in PBS and then washed three times with PBS and once with water. Slides were mounted using Fluormount G. Images were collected on a Delta Vision Optical Sectioning microscope consisting of an Olympus (Tokyo, Japan) IX-70 microscope and a photometrics CH 350 cooled charge-coupled device camera. An Olympus oil immersion 60× objective was used to collect the images.

Animals. Male Sprague-Dawley rats (Harlan (Indianapolis, IN), 150 to 200 g at the time of testing, were maintained in a climate-controlled room on a 12-h light/dark cycle (lights on at 7:00 AM), and food and water were available ad libitum. Rats were housed in pairs for at least 2 days before use. All of the testing was performed in accordance with the policies and recommendations of the International Association for the Study of Pain and the National Institutes of Health Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and received approval from the Institutional Animal Care and Use Committee of the ACADIA Pharmaceuticals, Inc.

Paw Edema Assays. Drugs were administered to rats directly into the paw (intrapaw) in a vehicle of 25 μl of DMSO using a Hamilton syringe. The controls were given the same volume of vehicle as in test groups. Paw edema or extent of the edema thickness (foot pad) was measured in conscious rats 4 h after injections using a caliper and normalized to the width of the contralateral paw.

Thermal Hyperalgesia Assays. The method of Hargreaves et al. (1988) was used to assess paw withdrawal latency to a thermal nociceptive stimulus. Rats were placed in a clear acrylic box on a glass platform and allowed to acclimate. A constant-intensity radiant heat source (model no. 7371 Plantar Test; Ugo Basile, Comerio, Italy) was applied through the platform to the midplantar area of the hind paw. The response to the stimulus was recorded by a photocell detecting withdrawal of the paw, and the latency is expressed in seconds. To prevent tissue damage, stimulation to the paw was terminated if the rats failed to withdraw after 20 s. Drugs were dosed intraperitoneally or directly into the paw as indicated. A significant reduction in paw withdrawal latency from pretreatment baseline or vehicle-treated control values was interpreted as thermal hyperalgesia.

Pharmacokinetics. AC-55541 and AC-264613 were prepared at 10 mg/ml in DMSO. Three rats per compound were dosed intraperitoneally at 10 mg/kg. Plasma samples were collected at 0, 0.083, 0.176, 0.25, 0.5, 1, 2, 4, 6, and 24 h and analyzed by LC/tandem mass spectrometry. The LC/tandem mass spectrometry analysis was performed using a 4000 QTRAP (Applied Biosystems, Foster City, CA) hybrid triple quadrupole linear ion-trap mass spectrometer equipped with electrospray ionization and operated in multiple reaction monitoring mode. AC-55541 and AC-264613 ion pairs were 518.2/105 and 401.2/200, respectively. The mass spectrometer was coupled to a high-performance liquid chromatography system consisting of two Shimadzu (Kyoto, Japan) LC-20AD high-performance pumps interfaced with a Shimadzu CBM-20A controller and a CTC HPL (LEAP Technologies, Carrboro, NC) autosampler. Separation was performed using a 4.6 × 50-mm Phenomenex Synergi Fusion (Torrance, CA) reversed-phase C18 column equipped with a guard column. LC solvent A was water, and B was acetonitrile, each containing 1% formic acid. Data collection and processing were performed using Analyst software version 1.4.2 (Applied Biosystems). The pharmacokinetic parameters were calculated with WinNonlin 5.0 (Pharisrt, Mountain View, CA).

Results

Using a cellular proliferation assay (R-SAT) (see Burstein et al., 2006), we screened the human PAR2 receptor against a diverse chemical library derived from commercial and proprietary sources containing over 250,000 compounds. This compound library had a significant overlap in chemical space with the MDL Drug Data Report library of known drugs and was heavily weighted to compounds within a mol. wt. range suggested by standard drug-likeness rules (see Lipinski et al., 2001). Screening hits were chemically optimized, and two compounds called AC-55541 and AC-264613 (see Fig. 1), each suggested by standard drug-likeness rules (see Lipinski et al., 2001).
AC-55541  AC-264613

**Fig. 1.** Structures of AC-55541 and AC-264613.

2008), were characterized in detail. Both compounds obeyed the Lipinski rule-of-five (see Lipinski et al., 2001), with the exception that AC-55541 is slightly over mol. wt. 500 (AC-55541: mol wt., 518.37; logD, 4.65; H-bond donors, 3; H-bond acceptors, 8; AC-264613: mol. wt., 400.27; logD, 2.20; H-bond donors, 2; H-bond acceptors, 5). Thus, both compounds have drug-like properties.

Concentration-response experiments demonstrated that AC-55541 and AC-264613 each stimulated cellular proliferation in a PAR2-specific manner. To eliminate proteolysis as a contributing factor to receptor activation, the protease-insensitive mutant PAR2 S37P was used (Fig. 2A). The potencies of AC-55541 and AC-264613 in the cellular proliferation assay were approximately 200 and 50 nM, respectively, and were virtually the same at wild-type PAR2 receptors (Table 1). In comparison, the potencies of SLIGRL-NH₂ and 2-furyl-LIGRLO-NH₂ were approximately 10 μM and 25 nM, respectively, comparable with previous reports (Kawabata et al., 2004a; McGuire et al., 2004). Both AC-55541 and AC-264613 displayed essentially full efficacy compared with SLIGRL-NH₂ in this assay. Neither the compounds nor the PAR2 APs displayed activity in cells transfected with reporter alone (Fig. 2A).

To assess their receptor selectivity, we tested AC-55541 and AC-264613 on cells transfected with the other PAR subtypes. We observed no activity of either compound at cells transfected with the protease-insensitive mutants PAR1 S42P or PAR4 R47A (Fig. 2, B and C). PAR3 does not respond to soluble peptides derived from its tethered ligand; however, it has been shown to interact with and potentiate the responses to both PAR1 and PAR4 (Nakanishi-Matsui et al., 2000; McLaughlin et al., 2007). We observed that cotransfection of PAR3 receptors strongly potentiated the responses of PAR1 S42P and PAR4 R47A to SFLLRN-NH₂ and AYPGKF-NH₂, respectively, indicating PAR3 is functionally active in the cellular proliferation assay (Fig. 2, B and C). The potency of PAR1 was increased approximately 6-fold when PAR3 was coexpressed (pEC₅₀, 6.0 ± 0.1 for PAR1 plus PAR3 versus 5.2 ± 0.1 for PAR1 alone; p < 0.01), whereas the maximal response of PAR4 was increased 2.3 ± 0.3-fold (p < 0.01) when PAR3 was coexpressed. However, we observed no activity of either AC-55541 or AC-264613 at cells transfected with PAR1 S42P plus PAR3 or PAR4 R47A plus PAR3 or PAR3 alone (Fig. 2, B and C). Furthermore, neither compound had significant affinity for over 30 other targets implicated in nociception and inflammation including A1, A2A, A2B, and A3 adenosine receptors, B1 bradykinin receptors, CB1 and CB2 cannabinoid receptors, cholecystokinin B receptors, CGRP receptors, CXCR4 receptors, EP4 and FP prostanoid receptors, endothelin A receptors, N-formyl-t-leucyl-t-leucyl-t-phenylalanine receptors, H₁ and H₄ histamine receptors, LTB₄ and LTD₄ leukotriene receptors, quinuclidinyl benzilate-labeled muscarinic receptor binding sites and neuropeptide Y-labeled binding sites in rat cerebral cortex, NK₁ and NK₃ tachykinin receptors, naloxone-labeled binding sites in rat cerebral cortex, platelet-activating factor receptors, TRKA receptors, N-methyl-D-aspartate, K⁺ channel, Na⁺ channel (site 2), GABAA, Ca²⁺ channel (N),
TABLE 1
Pharmacological activity of PAR2 agonists

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Receptor</th>
<th>Cellular Proliferation</th>
<th>PI Hydrolysis</th>
<th>Ca(^{2+}) Mobilization</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>pEC(_{50}) Efficacy</td>
<td>pEC(_{50}) Efficacy</td>
<td>pEC(_{50}) Efficacy</td>
</tr>
<tr>
<td>AC-55541</td>
<td>WT</td>
<td>6.7 ± 0.1 81 ± 4</td>
<td>5.9 ± 0.1 96 ± 10</td>
<td>6.6 ± 0.1 82 ± 4</td>
</tr>
<tr>
<td></td>
<td>S37P</td>
<td>6.7 ± 0.3 78 ± 6</td>
<td>5.4 ± 0.2 110 ± 7</td>
<td>N.D.</td>
</tr>
<tr>
<td>AC-264613</td>
<td>WT</td>
<td>7.5 ± 0.1 93 ± 15</td>
<td>6.9 ± 0.1 88 ± 14</td>
<td>7.0 ± 0.2 110 ± 2</td>
</tr>
<tr>
<td></td>
<td>S37P</td>
<td>7.3 ± 0.2 88 ± 23</td>
<td>6.5 ± 0.1 100 ± 8</td>
<td>N.D.</td>
</tr>
<tr>
<td>2-f-LIGRLO-NH(_2)</td>
<td>WT</td>
<td>7.6 ± 0.1 89 ± 11</td>
<td>7.4 ± 0.1 82 ± 3</td>
<td>7.1 ± 0.1 103 ± 5</td>
</tr>
<tr>
<td></td>
<td>S37P</td>
<td>7.5 ± 0.2 91 ± 21</td>
<td>6.9 ± 0.0 96 ± 5</td>
<td>N.D.</td>
</tr>
<tr>
<td>SLIGRL-NH(_2)</td>
<td>WT</td>
<td>5.0 ± 0.3 100 ± 0</td>
<td>5.0 ± 0.1 100 ± 0</td>
<td>5.0 ± 0.1 100 ± 0</td>
</tr>
<tr>
<td></td>
<td>S37P</td>
<td>5.0 ± 0.3 100 ± 0</td>
<td>5.3 ± 0.3 100 ± 0</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not done; WT, human wild-type PAR2; S37P, protease-insensitive mutant of PAR2.

and P2X ion channels, and serotonin and norepinephrine transporters (data not shown).

PAR2 receptors stimulate production of inositol phosphates and release of intracellular Ca\(^{2+}\). Therefore, to verify the results obtained using the cellular proliferation assay, AC-55541 and AC-264613 were tested for agonist activity at PAR2 in PI hydrolysis assays and Ca\(^{2+}\) mobilization assays (see Figs. 3 and 4; Table 1). The potencies of SLIGRL-NH\(_2\) and 2-furoyl-LIGRLO-NH\(_2\) in the PI hydrolysis and Ca\(^{2+}\) mobilization assays were approximately 10 \(\mu\)M in both assays for SLIGRL-NH\(_2\) and approximately 100 nM in both assays for 2-furoyl-LIGRLO-NH\(_2\). In the PI hydrolysis assays, AC-55541 had potencies of approximately 1 to 3 \(\mu\)M, and AC-264613 had potencies of approximately 100 to 300 nM, respectively. In the Ca\(^{2+}\) mobilization assay, AC-55541 and AC-264613 had potencies of approximately 250 and 100 nM, respectively. To confirm the observation made above in the cellular proliferation assays that AC-55541 and AC-264613 were tested for agonist activity at the PAR1 and PAR4 receptors (Fig. 3, A–C). Robust responses in PI hydrolysis assays on cells transfected with control vec-

2

tor, PAR1, or PAR4 receptors (Fig. 3, A–C). Robust responses to the PAR1 and PAR4 APs were observed, with pEC\(_{50}\) values of 6.1 ± 0.1 for SFLLRN-NH\(_2\) on PAR1, and 4.5 ± 0.2 for AYPGKF-NH\(_2\) on PAR4 (Fig. 3C). However, little or no response to either AC-55541 (Fig. 3A) or AC-264613 (Fig. 3B) was observed, except for a slight effect of AC-55541 at high (>10 \(\mu\)M) concentrations. This slight effect was observed in all conditions including cells transfected with empty vector; therefore, it is probably not due to activation of either PAR1 or PAR4. Similarly, we observed no significant calcium signal in untransfected KNRK cells (data not shown), in agreement with previous observations that KNRK cells do not express functional PAR2 receptors (Al-Ani et al., 1999). In general, all of the compounds had full or nearly full efficacy compared with SLIGRL-NH\(_2\) in all the functional assays. The rank order of potencies of all tested ligands was consistent across all functional assays and was 2-furoyl-LIGRLO-NH\(_2\) > AC-264613 > AC-55541 > SLIGRL-NH\(_2\) (Table 1).

PAR2 receptors have been reported to rapidly internalize upon activation by trypsin or PAR2 APs (Déry et al., 1999). Using EYFP-tagged PAR2 S37P receptors transiently trans-
fected into HEK 293T cells, we examined the internalization behavior of PAR2 receptors exposed to either PAR2 APs or the small organic molecules described here. As shown in Fig. 5, receptor internalization could be visualized as a redistribution of fluorescence from a uniform border located around the periphery of the cells into punctate vesicles located inside the cells. All four ligands induced receptor internalization and vesicle formation. However, SLIGRL-NH\(_2\) appeared to induce this process slightly faster.

Poor bioavailability is a major limitation of PAR2 APs for in vivo experiments. Intraperitoneal administration of AC-55541 or AC-264613 resulted in peak plasma concentrations of 1035 and 1233 ng/ml at 50 and 10 min, respectively, in male Sprague-Dawley rats (Fig. 6; Table 2). The elimination half-lives of AC-55541 and AC-264613 were 6.1 and 2.5 h, respectively (Fig. 6; Table 2). In vitro testing revealed that both AC-55541 and AC-264613 are reasonably stable to metabolism in the presence of liver microsomes, with values of 6 and 19 \(\mu\)l/min/mg for AC-55541 and values of 9 and 37 \(\mu\)l/min/mg for AC-264613 in human and rat liver microsomes, respectively.

PAR2 receptors have been strongly implicated in nociceptive and inflammatory processes. Therefore, we characterized AC-55541, AC-264614, and the PAR2 APs in hot plate and paw edema assays, commonly used models of acute thermal nociception, and acute inflammation. Administration of SLIGRL-NH\(_2\) or trypsin into the paw elicited robust thermal hyperalgesia and edema in male Sprague-Dawley rats (Fig. 7, A and B), which persisted for a period of at least 4 h, the longest time point examined. This finding is consistent with previous reports (Vergnolle et al., 1999, 2001; Steinhoff et al., 2000). Similarly, intrapaw administration of AC-55541 (Fig. 7, C and D) or AC-264613 (Fig. 7, E or F) resulted in hind paw edema and thermal hyperalgesia at doses as low as 30 ng. The maximal effects of SLIGRL-NH\(_2\), trypsin, AC-55541, and AC-264613 on thermal hyperalgesia and edema were similar to each other (see Fig. 7) and to the maximal effects we have previously observed using carrageenan (data not shown).

PAR2 receptors have been reported to elicit pain and inflammation through a neurogenic mechanism of action, causing release of substance P, activation of NK1 receptors, and sensitization of TRPV1 receptors (Steinhoff et al., 2000; Vergnolle et al., 2001; Amadesi et al., 2004; Dai et al., 2004). Therefore, we tested the effects of L732,138, a selective NK1 receptor antagonist, and capsazepine, a TRPV1 receptor antagonist on the pronociceptive effects of AC-55541 and AC-264613. As shown in Fig. 8, both L732,138 and capsazepine each completely blocked the pronociceptive actions of AC-55541 and AC-264613.

We next examined the hyperalgesic effects of AC-55541 and AC-264613 in male Sprague-Dawley rats after systemic administration. As shown in Fig. 9, AC-55541 and AC-264613 each showed dose-dependent pronociceptive
We have described the discovery and characterization of the first small-molecule PAR2 agonists AC-55541 and AC-264613. Both compounds displayed high potency and efficacy for PAR2 receptors in a variety of functional assays including cellular proliferation, calcium mobilization, and PI hydrolysis assays, and both compounds stimulated internalization of PAR2 receptors (see Figs. 2–5). To assess the selectivity of AC-55541 and AC-264613, we tested them at PAR1 and PAR4 receptors, each expressed alone or together with PAR3. PAR3 does not respond to soluble peptides derived from its tethered ligand; however, in agreement with previous reports (Nakanishi-Matsui et al., 2000; McLaughlin et al., 2007), it significantly potentiated the responses to both PAR1 and PAR4 (see Fig. 2, B and C). We observed no response to either AC-55541 or AC-264613 under any of these conditions. In terms of assay sensitivity, the potencies of the PAR1, PAR2, and PAR4 APs reported in our cellular proliferation assays and our PI hydrolysis assays are very similar to their potencies reported in other systems (Lerner et al., 1996; Kawabata et al., 2004a; McGuire et al., 2004; Quinton et al., 2004). Thus, our assessment is that these compounds are highly selective for PAR2 and would be expected to show similar selectivity in systems commonly employed in other laboratories. Ultimately, it will be important to test them in native systems with each of the PAR subtypes deleted to fully characterize their selectivity in vivo. Both compounds were metabolically stable, were well absorbed systemically, and remained at effective plasma concentrations for several hours, with AC-55541 achieving more sustained exposure but AC-264613 maintaining higher initial exposure (see Table 2).

Injection of PAR2 APs into the rat paw has been shown previously to cause a strong acute inflammatory response characterized by marked edema and granulocyte infiltration (Vergnolle et al., 1999). Consistent with their profiles as PAR2 agonists, both AC-55541 and AC-264613 produced robust hyperalgesia and edema in rats, either administered locally (intrapaw) or systemically (intraperitoneally) (see Figs. 7 and 9). AC-55541 produced more robust effects in vivo, especially when given systemically (see Fig. 9), despite the fact that it is less potent than AC-264613 in vitro (Table 1), possibly due to its more sustained exposure (see Fig. 6). Howev-

Ample evidence indicates PAR2 receptors mediate prono-
induced currents, and antagonism or deletion of TRPV1 receptors blocks PAR2-induced thermal hyperalgesia (Amadesi et al., 2004; Dai et al., 2004). Consistent with their actions as PAR2 agonists, we observed that thermal hyperalgesia induced by AC-55541 or AC-264613 was completely blocked by either the NK1 antagonist L732,138 or the TRPV1 antagonist capsazepine (see Fig. 8).

The possible therapeutic utility of PAR2 agonists is controversial, although therapeutic roles for PAR2 agonists have been proposed (De Campo and Henry, 2006; Henry, 2006). PAR2 activation has been shown to cause airway smooth muscle relaxation (Cicala et al., 2001), primarily in an epithelium-derived, PGE2-dependent manner (Cocks et al., 1999; Kawabata et al., 2004; De Campo and Henry, 2005), and by CGRP and PGE2 (Morello et al., 2005); however, other studies have demonstrated that PAR2 agonists may also cause bronchoconstriction (Schmidlin et al., 2002) through a neural mechanism (Su et al., 2005). One study showed PAR2 activation caused relaxation of trachea and main bronchi in an epithelium-derived, PGE2-dependent manner but constriction of intrapulmonary bronchi in a tachykinin receptor-dependent manner (Ricciardolo et al., 2000). Interestingly, PAR2 agonists potentiate histamine-induced contractions of human isolated bronchial rings from nonsmokers but antagonize histamine-induced contraction in isolated bronchial rings from smokers (Risse et al., 2004). More recently, it was shown that activation of PAR2 reduced airway inflammation and airway hyper-responsiveness in rabbits sensitized to pollen allergen (D’Agostino et al., 2007). These authors speculated that the use of pollen allergen instead of ovalbumin as the sensitizing agent may represent an asthma model more relevant to the human condition. Thus, the net effect of PAR2 activation on lung airflow may depend on species differences, anatomical differences (trachea and main bronchi versus deeper bronchioles), differences in the inflammatory state of the lungs, differences in the sensitizing agent, and differences in route of administration, which could affect exposure of the ligand to neurons versus epithelium.

Similarly, single high doses of PAR2 agonists inhibit inflammation of inflammatory cells into the lungs and reduce airway resistance of lipopolysaccharide-challenged mice and ovalbumin sensitized mice (Moffatt et al., 2002; De Campo and Henry, 2005; Morello et al., 2005), whereas administration of multiple doses of SLIGRL-NH₂ or genetic overexpres-
sion of PAR2 increased airway hyperresponsiveness, infiltration of eosinophils and mononuclear cells, and levels of proinflammatory cytokines (Schmidlin et al., 2002; Ebeling et al., 2005). Thus, PAR2 receptors may play a dual role in the lung.

In both HCl- and indomethacin-induced models of gastric...
ulcer PAR2 agonists prevent/limit tissue damage to the stomach lining by inducing mucus secretion, possibly through a neurogenic mechanism (Kawabata et al., 2005). Likewise, cerulein-induced pancreatitis and associated abdominal hyperalgesia/allodynia were exacerbated in PAR2 knockout mice and suppressed by 2-furoyl-LIGRLO-NH2 in WT, but not PAR2, knockout mice (Kawabata et al., 2006). PAR2 has been implicated as proinflammatory in animal models of colitis, pancreatitis, and in the development and progression of inflammatory bowel disease in humans (Nguyen et al., 2003; Hansen et al., 2005; Cenac et al., 2007; Hyun et al., 2008; Laukkarinen et al., 2008).

The identification of potent, selective, and metabolically stable small-molecule PAR2 ligands offers a number of new avenues for further exploration, including use in chronic studies, the development of radiolabeled probes for use in medical diagnostics and for binding studies, and development of antagonist analogs. Besides AC-264613 and AC-55541, we have successfully identified a series of active structural analogs of both compounds (see Seitzberg et al., 2008), demonstrating that these scaffolds are amenable to chemical modification. Given the controversial but clearly crucial role PAR2 receptors play in a wide variety of conditions with a strong inflammatory component and the substantial number of published studies indicating possible therapeutic roles for PAR2 agonists (discussed above), it will be important to further test the compounds described herein in those models.

Fig. 8. PAR2 agonists cause hyperalgesia through a neurogenic mechanism. Thermal hyperalgesia was measured as described above. One hour before intraperitoneal administration of 10 µg of either AC-55541 or AC-264613, male Sprague-Dawley rats were dosed with 10 mg/kg i.p. of the NK1 receptor antagonist L732,138, the TrpV1 receptor antagonist capsazepine, or saline where indicated. A, AC-55541 and L732,138; B, AC-264613 and L732,138; C, AC-55541 and capsazepine; D, AC-264613 and capsazepine. Data shown were taken at 2 h postdose of the antagonists (1 h after dose of AC-55541 or AC-264613) and are the means ± S.E.M. of six animals per group. **, p < 0.01 compared with vehicle.

Fig. 9. Systemic actions of PAR2 agonists. Male Sprague-Dawley rats were dosed i.p. with the indicated doses (milligrams per kilogram) of either AC-55541 (A) or AC-264613 (B), and thermal hyperalgesia was measured as described above at the indicated time points. Data shown are the means ± S.E.M. of six to 12 animals per group. *, p < 0.05 compared with vehicle. **, p < 0.01 compared with vehicle.

References


