Potent and Metabolically Stable Agonists for Protease-Activated Receptor-2: Evaluation of Activity in Multiple Assay Systems in Vitro and in Vivo

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ABSTRACT

To develop potent and metabolically stable agonists for protease-activated receptor-2 (PAR-2), we prepared 2-furoylated (2f) derivatives of native PAR-2-activating peptides, 2f-LIGKV-OH, 2f-LIGRL-OH, 2f-LIGKV-NH₂, and 2f-LIGRL-NH₂, and systematically evaluated their activity in PAR-2-responsive cell lines and tissues. In both HCT-15 cells and NCTC2544 cells overexpressing PAR-2, all furoylated peptides increased cytosolic Ca²⁺ levels with a greater potency than the corresponding native peptides, although a similar maximum response was recorded. The absolute potency of each peptide was greater in NCTC2544, possibly due to a higher level of receptor expression. Furthermore, the difference in potency between the 2-furoylated peptides and the native peptides was enhanced when evaluated in the rat superior mesenteric artery and further increased when measuring PAR-2-mediated salivation in ddY mice in vivo. The potency of 2f-LIGRL-NH₂, the most powerful peptide, relative to SLIGKV-OH, was about 100 in the cultured cell Ca²⁺ signaling assays, 517 in the vasorelaxation assay, and 1100 in the salivation assay. Amastatin, an aminopeptidase inhibitor, augmented salivation caused by native peptides, but not furoylated peptides. The PAR-2-activating peptides, including the furoylated derivatives, also produced salivation in the wild-type C57BL/6 mice, but not the PAR-2-deficient mice. Our data thus demonstrate that substitution of the N-terminal serine with a furoyl group in native PAR-2-activating peptides dramatically enhances the agonistic activity and decreases degradation by aminopeptidase, leading to development of 2f-LIGRL-NH₂, the most potent peptide. Furthermore, the data from PAR-2-deficient mice provide ultimate evidence for involvement of PAR-2 in salivation and the selective nature of the 2-furoylated peptides.

Protease-activated receptors (PARs) belong to a large superfamily of G-protein-coupled seven transmembrane domain receptors, mediating cellular actions of specific serine proteases (Macfarlane et al., 2001). Among four PAR family members cloned to date, PAR-1, PAR-2, and PAR-4 can be activated through enzymatic and nonenzymatic mechanisms (Vu et al., 1991; Nystedt et al., 1994; Kahn et al., 1998), although activation of PAR-3 is achieved only by the former mechanism (Ishihara et al., 1997; Xu et al., 1998). In enzymatic activation, the cryptic receptor-activating sequence (e.g., SLIGKV—and SLIGRL—for human and mouse/rat PAR-2, respectively) present in the N-terminal extracellular domain, after enzymatic unmasking, binds to the body of the same receptor molecule as a tethered ligand, leading to intracellular signaling through G proteins, especially Go11. Nonenzymatic receptor activation is achieved by direct binding of exogenously applied synthetic peptides based on the tethered ligand sequence to the body of the receptor (Macfarlane et al., 2001). Thus, structural modification of the receptor-activating peptides might lead to development of more potent agonists than the original peptide and hopefully antagonists.

ABBRVIATIONS: PAR, protease-activated receptor; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; HUVEC, human umbilical vein endothelial cell; FCS, fetal calf serum; RT-PCR, reverse transcriptase-polymerase chain reaction; bp, base pair; TFA, trifluoroacetic acid.
PAR-1, PAR-3, and PAR-4 are thrombin receptors, mediating platelet activation in distinct species (e.g., PAR-1 and PAR-4 in human platelets, and PAR-3 and PAR-4 for mouse/rat platelets) (Vu et al., 1991; Ishihara et al., 1997; Kahn et al., 1998; Xu et al., 1998; Macfarlane et al., 2001). PAR-2 is a receptor for trypsin, trypstatin, and coagulation factors VIIa and Xa, but not thrombin (Nystedt et al., 1994; Kawabata et al., 2001c; Macfarlane et al., 2001; Kawabata, 2003a). A number of studies using specific receptor-activating peptides for PAR-2 (e.g., SLIGRL-NH₂) and PAR-2-knockout mice have identified various physiological/pathophysiological roles played by PAR-2. PAR-2 seems to play a dual role in the inflammatory process, being pro- and anti-inflammatory (Steinhoff et al., 2000; Fiorucci et al., 2001; Cenac et al., 2002; Moffatt et al., 2002). There is also evidence that PAR-2 present in sensory neurons (Steinhoff et al., 2000), upon activation, causes pain sensation and hyperalgesia (Kawabata et al., 2001a, 2002a, b; Vergnolle et al., 2001). Specifically in the gastrointestinal tract, PAR-2 plays extensive roles, modulating gastric mucosal functions (Kawabata et al., 2001b; Kawao et al., 2002; Nishikawa et al., 2002; Kawabata, 2003a,b); salivary and pancreatic exocrine secretion (Nguyen et al., 1999; Kawabata et al., 2000a, b; Kawabata et al., 2002c). Together, PAR-2 could be a novel target for drug development, and both agonists and antagonists might be therapeutically available for treatment of various diseases.

SLIGKV-OH and SLIGRL-OH are original PAR-2-activating peptides derived from human and mouse/rat PAR-2, respectively. Their amidated peptides SLIGKV-NH₂ and SLIGRL-NH₂ are more potent than the original peptides. Interestingly, the agonistic activity of SLIGRV-NH₂ is higher than SLIGKV-NH₂ for mouse/rat and even human PAR-2 (Hollenberg et al., 1996, 1997; Kawabata et al., 2000b). Most recently, 2-furoyl-Leu-Ile-Gly-Arg-Leu-amide (2f-LIGRL-NH₂) was developed as a potent PAR-2 agonist (Ferrell et al., 2003). The agonistic activity of 2f-LIGKV-OH in vitro and in vivo is not dramatically higher than SLIGKR-NH₂, whereas intra-articular injection of 2f-LIGKV-OH in vitro and in vivo is not dramatically higher than SLIGRL-NH₂, whereas intra-articular injection of 2f-LIGKV-OH reveals prolonged proinflammatory effects, compared with SLIGRK-NH₂ in vivo (Ferrell et al., 2003). The longer duration of the effect of 2f-LIGKV-OH might be due to a slow rate of metabolic degradation. In the present study, in addition to 2f-LIGKV-OH, we prepared derivatives of native PAR-2-activating peptides, SLIGKV-OH, SLIGRL-NH₂, and SLIGRK-NH₂, in which the N-terminal serine residue was substituted with a furoyl group and evaluated their agonistic activities in vitro and in vivo systematically. Three distinct assay systems were used: 1) Ca²⁺ signaling in human cultured cells that naturally express PAR-2 or overexpress PAR-2 by gene transfection; 2) relaxation/contraction responses in rat superior mesenteric artery; 3) in vivo salivation in ddY mice and in the wild-type (PAR-2²/²) and PAR-2-deficient (PAR-2⁻/⁻) C57BL/6 background mice. Here, we show that 2f-LIGRK-NH₂ is the most potent PAR-2 agonist, especially in vivo, and resistant to metabolic degradation with aminopeptidase.

Materials and Methods

Test Chemicals. The PAR-2-activating peptides used were as follows: Ser-Leu-Ile-Gly-Lys-Val (SLIGKV-OH), Ser-Leu-Ile-Gly-Arg-Leu (SLIGRL-OH), Ser-Leu-Ile-Gly-Lys-Val-amide (SLIGKV-NH₂), Ser-Leu-Ile-Gly-Arg-Leu-amide (SLIGRL-NH₂), 2-furoyl-Leu-Ile-Gly-Lys-Val (2f-LIGKV-OH, ASKH95), 2-furoyl-Leu-Ile-Gly-Arg-Leu (2f-LIGRL-OH), 2-furoyl-Leu-Ile-Gly-Lys-Val-amide (2f-LIGKV-NH₂), and 2-furoyl-Leu-Ile-Gly-Arg-Leu-amide (2f-LIGRL-NH₂). The PAR-1-activating peptide Thr-Ph-Arg-Arg-Val (TFLLR-NH₂) was also generated in the above experiments. All peptides were synthesized and purified by high-performance liquid chromatography (HPLC), and the concentration and purity were determined by mass spectrometry.

Animals. Male Wistar rats (7 weeks old) and male ddY mice (22-28 g) were purchased from Japan SLC Inc. (Shizuoka, Japan). The PAR-2-deficient mice, derived from C57BL/6 background, were generated as described previously (Ferrell et al., 2003). The PAR-2-deficient strain was maintained by backcrossing heterozygous (PAR-2²/⁻) male with C57BL/6 females at each generation at Kowa Tokyo New Drug Research Laboratories (Tokyo, Japan). The genotype of the mice was confirmed by Southern blot analysis and PCR analysis of DNA obtained from tail biopsy. Homozygous (PAR-2⁻/⁻) and wild-type (PAR-2²/²) female mice generated from male and female PAR-2⁻/⁻ mice at backcross generation eight were used at 8 to 12 weeks of age for the experiments. All animals were used with approval by the Kinki University School of Pharmaceutical Science’s Committee for the Care and Use of Laboratory Animals.

Cell Cultures. The human umbilical vein endothelial cells (HUVECs) obtained from Cambrex Bio Science Baltimore, Inc. (Baltimore, MD) were cultured in the complete endothelial cell growth medium (Cambrex Bio Science Baltimore, Inc.) containing bovine brain extract (12 μg/ml), human endothelial growth factor (10 ng/ml), hydrocortisone (1 μg/ml), fetal calf serum (FCS) (2%), and gentamicin (50 μg/ml) plus amphotericin-B (50 ng/ml). The cells were passaged with trypsin and used at passages 4 to 8 for the experiments. During the period of passages 4 to 8, no noticeable change in expression levels of functional PAR-2 was observed in the preliminary experiments. The human colorectal adenocarcinoma cell line HCT-15 was obtained from American Type Tissue Culture Collection (Manassas, VA) (ATCC CRL 1569) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FCS, sodium bicarbonate (50 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The human skin epithelial cell line NCTC2544 that expresses human PAR-2 was prepared, as described previously (Kanke et al., 2001). A plasmid encoding human PAR-2 (PAR-2pRC/RSV) was transfected into NCTC2544 cells using Lipofect (Invitrogen, Carlsbad, CA), and geneticin-resistant clones were isolated by limited dilution. A clone that stably expressed PAR-2 was selected by determining PAR-2-mediated [³⁵S]inositol phosphate accumulation and designated as NCTC2544-PAR-2 cells. The background NCTC2544 cells were grown in medium without geneticin. Early-salts containing FCS (10%), sodium bicarbonate (50 mM), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The NCTC2544-PAR-2 cells were maintained in the complete medium containing geneticin (400 μg/ml) for keeping selection pressure. NCT-15 and NCTC2544 cells were passaged using Versene (0.53 mM EDTA in phosphate-buffered saline) to avoid trypsin exposure. All cells were grown at 37°C in an incubator with saturated humidity and 5% CO₂.

Measurement of Ca²⁺ Mobilization. PAR-2 agonist-stimulated intracellular Ca²⁺ mobilization was measured in HCT-15, NCTC2544, and NCTC2544-PAR-2 cells, by the modified fluorescence technique (Hawthorne et al., 2001), using a 96-well scanning fluorometer (FLEXStation; Molecular Devices, Sunnyvale, CA). Appropriate numbers of cells (HCT-15, 50,000; and NCTC2544 and NCTC2544-PAR-2, 30,000 cells/well) were seeded in black-well clear-bottom 96-well plates (Corning Glassworks, Corning, NY) 24 h before the assay. The confluent cells were washed once with a serum-free medium, and 80 μl of the same medium was added to the wells. After addition of 80 μl of a calcium assay solution (FLEXstation Calcium Plus assay kit; Molecular Devices) dissolved in Hanks’ balanced salt solution (pH 7.4) containing 20 mM HEPES and 2.5 mM proline, the plates were incubated for 60 min at 37°C. The cells were stimulated with various concentrations of agonists prepared in Hanks’ balanced salt solution, and fluorescence change was moni-
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tored at 25°C (excitation wavelength, 485 nm; and emission wave-
length, 525 nm). Agonist-induced Ca\textsuperscript{2+} signals are expressed as the percentage of the reference Ca\textsuperscript{2+} response induced by a calcium ionophore, A23187, 10 μM.

**Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).** The levels of mRNA for PARs expressed in HCT-15, HUVEC, NCTC2544, and NCTC2544-PAR-2 cells were determined by quantitative RT-PCR. Total cellular RNA was isolated from cells using the RNaseasy Mini kit (QIAGEN Sciences Inc., Germantown, MD) and reverse transcribed with MuLV reverse transcriptase following manufacturer’s instructions (Applied Biosystems, Foster City, CA) using Random Hexamers. Quantitative PCR was performed with an ABI Prism 7900HT sequence detection system (Applied Biosystems). Oligonucleo-
tide primers and probes for human PARs 1 to 4 were designed using the Primer Express program and synthesized by Applied Biosystems. Specific primer pairs were used in combination with internal probes, each primer labeled at the 5’ end with the quencher dye 6-carboxyfluorescein and at the 3’ end with the quencher dye 6-carboxytetramethylrhodamine. The sequences of primers were as follows: 5’-TTGCTTTGCGACCA-
CAAA-3’ (sense) and 5’-CTCTGTGGTGGAGGTGTG-3’ (antisense) for human PAR-1; 5’-GGCCACATGTACTGTTCCATCT-3’ (sense) and 5’-GGTGCTGCTGCTGCTG-3’ (antisense) for human PAR-2; 5’-GGCGCCACGTCTTCTTCTAG-3’ (sense) and 5’-GCGGTATGCTGATGACCA-3’ (antisense) for human PAR-3; and 5’-CTGCGTGGATCTCCTC-3’ (sense) and 5’-CGTCTGCGCCA-
CCTTGT-3’ (antisense) for human PAR-4. The original sequences of the labeled probes were as follows: 5’-TTCCTCTGTGGAGGTGTG-3’ for PAR-1; 5’-TCATTCACTGCTGCTG-3’ for PAR-2; 5’-CAACTGTACTGTTCCATCT-3’ for PAR-3; and 5’-TATCCTGCGCCAAGTCTCC-3’ for PAR-4. Primers and probes for ribosomal RNA (rRNA) as an internal standard were purchased from Applied Biosystems. PCR reaction for each PAR and for rRNA were performed in triplicate in 25 μl of a reaction solution containing 200 nM TaqMan probe, 1× TaqMan Universal Master Mix (Applied Biosystems), and specific primer pairs. Amplification was performed for 40 cycles of 95°C for 15 s and 60°C for 60 s. Sequence-specific amplification was monitored as a real time increase in fluorescent signals. The sequence-specific mRNA level for each PAR was quantified using appropriate standard curves, and normalized by rRNA levels.

To confirm the absence of PAR-2 mRNA in the salivary glands of the PAR-2-deficient mice, conventional RT-PCR was performed. The PAR-2-/- and PAR-2-/- C57BL/6 background mice were killed by exanguination under urethane anesthesia, and the bilateral sublingual glands, known to express abundant PAR-2 mRNA in ddY mouse (Kawabata et al., 2000a), were excised. Total RNA was extracted from the tissue homogenate in the TRIzol reagent (Invitrogen) and reverse transcribed and then amplified by PCR using the RNA LA PCR kit (AMV) version 1.1 (Takara, Kyoto, Japan). The PCR primers were as follows: 5’-GGTCTGTACACGTCCATCAT-3’ (sense) and 5’-AGCCAAAGATCTGCTAGGTT-3’ (antisense) for mouse PAR-1; 5’-CAACAGTAAAGGAAGAATGCT-3’ (sense) and 5’-AGGCCAGACATCTGGCAGGT-3’ (antisense) for mouse PAR-2; and 5’-AACACGAGAAGCCATCATGAA-3’ (sense) and 5’-TGCATCTGGCACCAACACT-3’ (antisense) for mouse PAR-3. The PCR reactions for PARs and GAPDH were allowed to proceed for 35 and 30 cycles, respectively (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s). The PCR products (302 bp for PAR-1, 601 bp for PAR-2, and 259 bp for GAPDH) were visualized by 2% agarose gel electrophoresis followed by the ethidium bromide staining.

**Relaxation/Contraction Bioassay in Isolated Rat Superior Mesenteric Artery.** The rat was sacrificed by decapitation under urethane (1.5 g/kg i.p.) anesthesia, and the superior mesenteric artery was removed. The endothelium-intact and -denuded ring segments (0.5 mm in diameter, 3 mm in length) were prepared in an ice-cooled Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 25 mM NaHCO\textsubscript{3}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, and 10 mM glucose), and suspended in organ baths containing 2 ml of Krebs-Henseleit solution maintained at 37°C and bubbled with 95% O\textsubscript{2}, 5% CO\textsubscript{2} to keep the pH at 7.4. The segment was equilibrated for 1 h under a resting tension of 10 mN, and isometric tension was recorded through a force-displacement transducer (UL-106R; Minebea Co., Ltd., Kanagawa, Japan). The intraluminal pressure of the ring segment was monitored a few times by measuring the con-
tractile response to a high K\textsuperscript{+} (50 mM)-containing Krebs-Henseleit solution in which the corresponding molar equivalent of NaCl was removed. Relaxation responses were monitored in the endothelium-intact arterial ring precontracted with 1 μM phenylephrine, and the contractile activities were tested in the endothelium-free ring seg-
ment without preconstriction. The contractile responses are ex-
pressed as a percentage (% KCl) of the contraction induced by 50 mM K\textsuperscript{+}, and the relaxation responses are represented as a percentage (% papaverine) of the relaxation to 100 μM papaverine.

**Salivation Bioassay in Anesthetized Mice in Vivo.** Salivary exocrine secretion was assessed in ddY mice and in PAR-2-/- and PAR-2-/- C57BL/6 background mice under urethane anesthesia, as described previously (Takeda and Krause, 1989; Kawabata et al., 2000b). Cotton was placed in the mouth for 1- or 5-min interval and repeatedly replaced with new cotton every 1 or 5 min. The difference of the weight of cotton before and after each interval was defined as the amount of secreted and absorbed saliva for each interval. Saliv-
ation was monitored for 20 min after i.v. challenge with agonists. In some experiments, amastatin (Peptide Institute, Minoh, Japan), an inhibitor of aminopeptidase, a degradation enzyme for peptides, was administered iv. i.m. immediately before agonists.

**Aminopeptidase Degradation of PAR-2-Activating Peptides in Vitro.** To determine the extent of aminopeptidase degradation of SLIGRL-NH\textsubscript{2} and 2F-LIGRL-NH\textsubscript{2}, the peptides at 50 μg/ml were incubated at 0, 15, 30, 45, 60 min in 0.1 M phosphate buffer (pH 7.4) containing 0.2 U/ml aminopeptidase (from Aeromonas proteolytica; Sigma-Aldrich, St. Louis, MO) in the absence or presence of 2.5 mM amastatin. The sample (25 μl) was then mixed with 10 μg/ml benzoic acid (10 μl) as an internal standard and 1.2% trifluoroacetic acid (TFA) (25 μl). After centrifugation, the superna-
tant was analyzed by the HPLC method. Briefly, HPLC analysis was carried out on an isocratic system (HPLC pump model LC-6AD; Shimadzu, Kyoto, Japan) with a COSMOSIL 5C\textsubscript{18}-AR-II column (150 × 4.6 mm; Nacalai Tesque, Kyoto, Japan) and an SPD-10A UV-Vis detector (Shimadzu) at a flow rate of 1.0 ml/min. The column temperature was 35°C. The isocratic mobile phase consisted of MeCN/TFA/water [200:1:700 (v/v)] and UV detection at 220 nm was used for determination of SLIGRL-NH\textsubscript{2}, whereas the alternative mobile phase consisted of MeCN/TFA/water [200:1:700 (v/v)] and UV detection at 215 nm was used for 2F-LIGRL-NH\textsubscript{2}. Quantification of the peptides was made using standard curves with authentic peptides, on the basis of the absolute peak areas.

**Statistics.** Data are represented as mean ± S.E.M. Statistical significance was analyzed by Student’s t test for two-group data and by Bonferroni’s test for comparison of two curves. Significance was set at P < 0.05 level.

**Results**

**Structure-Activity Relationships of PAR-2-Activating Peptides in HCT-15 cells as Assessed by Ca\textsuperscript{2+} Signaling.** We first determined levels of mRNA for PARs expressed in HCT-15 cells, compared with HUVECs known to functionally expresses PAR-1 and PAR-2 that, upon activation, induce cytosolic Ca\textsuperscript{2+} mobilization (Mirza et al., 1996; Molino et al., 1997). Quantitative RT-PCR analysis revealed that HCT-15 cells expressed PAR-2 mRNA equivalent to that in HUVECs, whereas expression levels of mRNA for PAR-1, PAR-3, and PAR-4 in HCT-15 cells were relatively negligible in comparison with HUVECs (Fig. 1A). The human PAR-2-derived receptor-activating peptide SLIGKV-OH at 1 to 1000 μM produced cytosolic Ca\textsuperscript{2+} mobilization in a concentration-
dependent manner (Fig. 1, B and C). All other PAR-2-activating peptides, SLIGRL-OH, SLIGKV-NH₂, SLIGRL-NH₂, 2f-LIGKV-OH, 2f-LIGRL-OH, 2f-LIGKV-NH₂, and 2f-LIGRL-NH₂, also evoked concentration-dependent Ca²⁺ signals in various concentration ranges (Fig. 1C). Although the magnitude of maximal responses to those peptides was almost the same, the potency of peptides in terms of the effective concentration range was greatly different. The order of the agonistic potency of the peptides was 2f-LIGRL-NH₂ > 2f-LIGKV-NH₂ > 2f-LIGRL-OH > 2f-LIGKV-OH > SLIGRL-NH₂ > SLIGKV-NH₂ > SLIGRL-OH > SLIGKV-OH (Fig. 1C). Of note was that thrombin, an activator of PAR-1, PAR-3, and PAR-4, at 3 U/ml caused a small response (approximately 44.9% of the response to A23187), although a larger dose, 10 U/ml, of thrombin did not cause further effect (data not shown).

**Ca²⁺ Signals Caused by PAR-2-Activating Peptides in NCTC2544-PAR-2 Cells.** We next evaluated the effects of the test compounds in the NCTC2544-PAR-2 cells. The background NCTC2544 cells expressed very low levels of mRNAs for PAR-1, PAR-2, PAR-3, and PAR-4, whereas the NCTC2544-PAR-2 cells expressed abundant PAR-2 mRNA (Fig. 2A). The PAR-2-activating peptides used evoked concentration-dependent cytosolic Ca²⁺ mobilization in NCTC2544-PAR-2 cells at concentrations lower than those in HCT-15 cells (Figs. 1C and 2B). This may be attributable to the difference in the abundance of PAR-2 between NCTC2544-PAR-2 and HCT-15 cells. The order of the potency of the peptides was as follows: 2f-LIGRL-NH₂ > 2f-LIGKV-NH₂ > 2f-LIGRL-OH > 2f-LIGKV-OH > SLIGRL-NH₂ > SLIGKV-NH₂ > SLIGRL-OH > SLIGKV-OH. In this assay system, the activity of 2f-LIGKV-OH (ASKH95) was thus lower than that of the native peptide SLIGRL-NH₂. None of the above-mentioned peptides evoked a significant Ca²⁺ response in NCTC2544 cells when used at concentrations close to the EC₅₀ value; the values of calcium mobilization (%A23187) evoked by SLIGRL-OH (10 μM), SLIGKV-OH (3

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**Fig. 1.** Expression levels of mRNA for PARs and agonist-induced intracellular Ca²⁺ mobilization in HCT cells. A, quantitative RT-PCR analysis of PARs in HCT-15 cells and HUVECs. Relative expression levels of mRNA for PARs were determined by real-time PCR (TaqMan assay) using 6-carboxyfluorescein-labeled PAR probes and a 6-VIC-labeled ribosomal RNA probe as an endogenous reference. TaqMan analysis was performed in triplicate, and the results are shown as the mean ± S.E.M. of three independent experiments. B and C, HCT-15 cells loaded with fluorescent Ca²⁺ indicator dye in 96-well plates were challenged with various concentrations of agonist peptides. Representative traces of fluorescence changes induced by SLIGKV-OH (1–1000 μM) and a Ca²⁺ ionophore, A23187 (10 μM), are presented in B. Peak fluorescence changes induced by a variety of agonist peptides with various concentrations were normalized by the maximal response mediated by A23187 (10 μM) (C). Data are expressed as the mean ± S.E.M. of triplicate experiments.
the relaxant activity of the peptides was as follows: 2f-LIGRL-NH₂, 2f-LIGKV-NH₂, SLIGRL-NH₂, and 2f-LIGKV-OH (1 μM), SLIGKV-NH₂ (1 μM), SLIGRL-NH₂ (1 μM), 2f-LIGKV-OH (1 μM), 2f-LIGRL-OH (1 μM), 2f-LIGKV-NH₂ (0.3 μM), and 2f-LIGRL-NH₂ (0.3 μM) were 2.2 ± 1.9, 0.0 ± 0.0, 0.0 ± 0.0, 0.8 ± 0.9, 1.2 ± 0.9, 1.2 ± 0.8, 2.0 ± 1.2, and 5.2 ± 2.7, respectively (n = 3). Furthermore, thrombin at up to 10 U/ml did not produce any response in the NCTC2544 cells (data not shown). Together, the Ca²⁺ signals caused by these peptides seem to occur via activation of PAR-2 in the NCTC2544-PAR-2 cells. We thus found three novel potent agonists possibly specific for PAR-2, 2f-LIGRL-NH₂, 2f-LIGKV-NH₂, and 2f-LIGRL-OH the agonistic activities of which were even greater than 2f-LIGKV-OH (ASKH95) that was previously designated ASKH95 (Ferrell et al., 2003).

**Relaxation/Contractile Activity of PAR-2-Activating Peptides in Rat Superior Mesenteric Artery.** In the ring preparations of rat superior mesenteric artery, the PAR-2-activating peptide SLIGRL-NH₂, but not the PAR-1-activating peptide TFLLR-NH₂, at 100 μM evokes endothelium-dependent relaxation responses, and the underlying mechanisms involve both endothelial nitric oxide and endothelium-derived hyperpolarizing factor (unpublished data). All PAR-2-activating peptides tested caused relaxation responses in the endothelium-intact arterial rings. The order of the relaxant activity of the peptides was as follows: 2f-LIGRL-NH₂ > 2f-LIGKV-NH₂ > 2f-LIGKV-OH > SLIGRL-NH₂ > SLIGKV-NH₂ > SLIGRL-OH > SLIGKV-OH (Fig. 3A). To examine the agonistic activity of the PAR-2-activating peptides toward PAR-1, we tested their effects in the endothelium-denuded preparation without precontraction, because activation of PAR-1, but not PAR-2, present in the smooth muscle of this artery causes contraction (unpublished data). Neither native peptides SLIGRL-OH and SLIGRL-NH₂ nor the furoylated compounds 2f-LIGKV-OH, 2f-LIGKV-NH₂, and 2f-LIGRL-NH₂ produced contraction in the preparation, although the PAR-1 agonist TFLLR-NH₂ at 10 to 100 μM caused concentration-dependent contractile responses (Fig. 3B), indicating the lack of agonistic activity of the furoylated peptides toward PAR-1.

**Structure-Activity Relationships of PAR-2-Activating Peptides for Salivation Responses in Anesthetized ddY Mice That Did or Did Not Receive Preadministration of Amastatin in Vivo.** All PAR-2-activating peptides tested, when administered i.v., caused prompt salivation in anesthetized ddY mice without preadministration of amastatin, and the order of the potency of the peptides in terms of the effective concentration range was as follows: 2f-LIGRL-NH₂ > 2f-LIGKV-NH₂ > 2f-LIGKV-OH > SLIGRL-NH₂ > SLIGKV-NH₂ > SLIGRL-OH > SLIGKV-OH. The maximal responses to the furoylated peptides were greater than those to the other peptides (Fig. 4A, top). The potency of SLIGKV-OH, SLIGRL-NH₂, and SLIGRL-NH₂ as secretagogues was greatly enhanced by combined administration with amastatin, and the maximum responses increased to that obtained for the substituted peptides. The facilitation by amastatin of the activity of SLIGRL-NH₂ was particularly great, its potency being even greater than 2f-LIGKV-OH (ASKH95) in the presence of amastatin (Fig. 4A). The time-related salivation responses to each peptide were compared in mice that did and did not receive amastatin, in which submaximal doses in the presence of amastatin were used (Fig. 4B). It is clear that amastatin significantly facilitated the effects of SLIGKV-OH, SLIGRL-NH₂, and SLIGRL-NH₂, but not the furoylated peptides. The effect of SLIGRL-OH was slightly enhanced by amastatin, whereas no significant facilitation was found (Fig. 4B).
Salivation Responses to the PAR-2-Activating Peptides in the PAR-2−/− and PAR-2+/+ C57BL/6 Background Mice. The absence of PAR-2 mRNA in the salivary gland of the PAR-2−/− mice was confirmed by RT-PCR analysis (Fig. 5A). The native PAR-2-activating peptide SLIGRL-NH₂ and the furoylated peptides 2f-LIGKV-OH (ASKH95) and 2f-LIGRL-NH₂, administered i.v. at maximal doses, 15, 5, and 0.15 μmol/kg, respectively, elicited prompt salivation in the PAR-2+/+ C57BL/6 mice (Fig. 5B), as in ddY mice. In contrast, all these PAR-2-activating peptides at the corresponding doses failed to cause salivation in the PAR-2−/− mice (Fig. 5B). Of note is that i.v. carbachol at 0.08 μmol/kg caused salivation, almost to the same extent, in the PAR-2+/+ and PAR-2−/− mice (Fig. 5B).

Distinct Aminopeptidase Degradation of 2f-LIGRL-NH₂ and SLIGRL-NH₂ in Vitro. The native PAR-2-activating peptide SLIGRL-NH₂ was actually greatly degraded by aminopeptidase in the absence, but not presence, of amastatin for 60 min (Fig. 6). In contrast, 2f-LIGRL-NH₂, the most potent PAR-2-activating peptide in vitro and in vivo, as described above, was resistant to aminopeptidase even in the absence of amastatin (Fig. 6).

Discussion

The present study systematically demonstrates that substitution of the N-terminal serine residue of native PAR-2-activating peptides with a furoyl group dramatically enhances the agonistic activity toward PAR-2, leading to development of 2f-LIGRL-NH₂, the most potent PAR-2 agonist among known peptides. Of note is that the high activity of the furoylated peptides is more conspicuous in vivo than in vitro. These profiles of the test compounds would be better understood by comparison of the EC₅₀ or ED₅₀ values and the relative potencies to the human-derived native peptide SLIGKV-OH (Table 1). The high activity of the furoylated peptides, particularly in vivo, can be in part explained by their metabolic stability, as suggested by the in vivo salivation study using amastatin and more directly by the experiments determining aminopeptidase degradation of peptides. Another important finding in our study is that salivary exocrine secretion was caused by all PAR-2-activating peptides in PAR-2+/+ C57BL/6 background mice, but not PAR-2−/− mice, providing ultimate evidence that PAR-2 actually mediates salivation and that these peptides are relatively selective for PAR-2.

The potency of the furoylated peptides relative to the activity of SLIGKV-OH in HCT-15 cells that naturally express PAR-2 was in parallel with that in human PAR-2-transfected NCTC2544 cells, although the EC₅₀ values of all test compounds were 25 to 68 times greater in the former cells than in the latter (Table 1). The higher activity of the compounds in the NCTC2544-PAR-2 cells is attributable to higher expression level of PAR-2, as indicated by the quantitative RT-PCR analysis. The possibility cannot be ruled out that Ca²⁺ signals caused by the test compounds in HCT-15 cells might include nonPAR-2 responses. In contrast, the Ca²⁺ mobilization evoked by the test compounds in NCTC2544-PAR-2 cells is entirely dependent on PAR-2 activation, considering that neither the PAR-2-activating peptides tested nor thrombin, an activator of PAR-1, PAR-3, and PAR-4, evoked responses in the background NCTC2544 cells. Of note is that the enhancement of the potency by furoylation of the PAR-2-activating peptides was more conspicuous in the rat superior mesenteric artery relaxation assay than in the cultured cell Ca²⁺ signaling assay, e.g., the potency of 2f-LIGRL-NH₂ relative to SLIGKV-OH was around 500 and 100 in the former and latter assay systems, respectively (Table 1). This inconsistency might be explained by two hypotheses: 1) the distribution/accessibility of the peptides to the PAR-2 receptor site in the arterial tissue might be improved by furoylation; and 2) the furoylation might make the peptides resistant to metabolic systems present in the arterial tissue, but not HCT-15 cells/NCTC2544-PAR-2 cells. It is of note that the endothelium-dependent vasorelaxation caused by the PAR-2 agonists in rat superior mesenteric artery does not involve PAR-1 activation, because PAR-1 agonists are incapable of producing relaxation response in this artery (unpublished data). Importantly, the lack of the agonistic activity of the furoylated peptides toward PAR-1 was demonstrated by the absence of the contractile activity in the endothelium-
Fig. 4. In vivo salivary exocrine secretion caused by PAR-2-activating peptides in anesthetized ddY mice. A, dose-related effects of the peptides as secretagogues in the absence [Ama (–)] or presence [Ama (+)] of coadministered amastatin. Each peptide was administered i.v. alone or immediately after i.v. amastatin at 2.5 μmol/kg, and the total amount of saliva secreted for 20 min was quantified. Data are expressed as the mean ± S.E.M. from four to seven mice. B, time-related effects of SLIGKV-OH at 50 μmol/kg, SLIGRL-OH at 50 μmol/kg, SLIGKV-NH₂ at 15 μmol/kg, SLIGRL-NH₂ at 1.5 μmol/kg, 2f-LIGKV-OH at 5 μmol/kg, 2f-LIGKV-NH₂ at 0.15 μmol/kg, and 2f-LIGRL-NH₂ at 0.15 μmol/kg, alone or in combination with amastatin. Each peptide was administered i.v. alone or immediately after i.v. amastatin at 2.5 μmol/kg, and the amount of saliva secreted every 5 min (or every 1 min; see bottom right) for 20 min was quantified. Data are expressed as the mean ± S.E.M. from four mice. ns, not significant (Bonferroni’s test).
denuded rat superior mesenteric arterial ring, in which PAR-1 agonists caused vasoconstriction. Of interest is that the potency of 2f-LIGKV-NH₂ and 2f-LIGRL-NH₂ relative to SLIGKV-OH in the in vivo salivation assay was even greater than that in the arterial relaxation assay. The dramatic facilitation and prolongation by furoylation of the activity as secretagogues in vivo can be interpreted, at least in part, by protection of the N-terminal of the peptides against metabolic degradation by aminopeptidase, because coadministered amastatin, an aminopeptidase inhibitor, potentiated and prolonged the effect of SLIGKV-NH₂ and SLIGRL-NH₂, but not 2f-LIGKV-NH₂ and 2f-LIGRL-NH₂. This is supported by our data from direct determination of aminopeptidase degradation of peptides in vitro. The reason remains unclear why amastatin produced only slight or no potentiation of the activity of SLIGKV-OH and SLIGRL-OH, peptides nonamidated at the C-terminal, as secretagogues. One possibility is that enzymes such as carboxypeptidase other than aminopeptidase might be more responsible for degradation of these peptides. The specificity of the furoylated peptides for PAR-2 as secretagogues could be demonstrated by the findings that salivary exocrine secretion was caused by 2f-LIGKV-NH₂ and 2f-LIGRL-NH₂, in addition to SLIGRL-NH₂, in the PAR-2⁻/⁻ C57BL/6 background mice, but not PAR-2⁻/⁻ mice, whereas carbachol-evoked salivation was retained in both mice.

In the in vivo salivation experiments, most peptides at supramaximal doses produced relatively reduced effects, resulting in bell-shaped dose-response curves. These characteristics are in agreement with the previous evidence (Kawabata et al., 2000b) and have yet to be investigated in detail. There is apparent discrepancy that introduction of a furoyl group into the peptide structure enhanced maximal responses in the in vivo salivation assay, but not in the in vitro Ca²⁺ signaling and arterial relaxation assays. One possibil-

Fig. 5. In vivo salivation activity of PAR-2-activating peptides in the PAR-2⁻/⁻ and PAR-2⁻/⁻ C57BL/6 background mice. A, RT-PCR analysis showing the presence of PAR-2 mRNA in the salivary gland of the PAR-2⁻/⁻ mice, but not PAR-2⁻/⁻ mice. B, SLIGRL-NH₂ at 15 μmol/kg, 2f-LIGKV-OH at 5 μmol/kg, 2f-LIGRL-NH₂ at 0.15 μmol/kg, or carbachol at 0.08 μmol/kg was administered i.v. to the PAR-2⁻/⁻ or PAR-2⁻/⁻ mice, and the total amount of saliva secreted for 20 min was quantified. Data are expressed as the mean ± S.E.M. of four mice. ns, not significant.

Fig. 6. Aminopeptidase degradation of SLIGRL-NH₂ and 2f-LIGRL-NH₂ in the absence and presence of amastatin. The peptides (50 μg/ml) were incubated with aminopeptidase (0.2 U/ml) for 0–60 min in the absence or presence of amastatin (2.5 μM). Data show the mean values of duplicate experiments.
ity is that the maximal activity in the two in vitro assay systems might not necessarily require full activation of PAR-2 receptors. However, it is more likely that the enhancement by furoylation of the maximal salivation responses to the PAR-2-activating peptides might reflect the aminopeptidase resistance of the furoylated peptides, which might be more important in vivo than in vitro. PAR-2 is now recognized as an important biological molecule, playing a variety of physiological/pathophysiological roles (Macfarlane et al., 2001; Kawabata, 2003a,b). Given the absence of specific PAR-2 antagonists, the use of PAR-2-activating peptides as research tools in vitro and in vivo has been critical to identify the functions of PAR-2. Combined administration of inhibitors of metabolic enzymes such as amastatin is necessary to examine the effect of native PAR-2-activating peptides such as SLIGKV-NH₂ and SLIGRL-NH₂ in some in vivo experiments (Kawabata et al., 2001b, 2003a,b). In this context, the furoylated peptides, especially 2f-LIGRL-NH₂, could serve as more powerful research tools to understand functions of PAR-2, in terms of the high potency and long duration of the effect that are partially attributable to resistance to aminopeptidase. Apart from possible involvement of PAR-2 in the pathogenesis of inflammatory diseases (Steinhoff et al., 2000; Macfarlane et al., 2001; Ferrell et al., 2003), PAR-2 also seems to play protective and/or anti-inflammatory roles in certain conditions in the alimentary and respiratory systems (Cocks and Moffatt, 2001; Fiorucci et al., 2001; Kawabata et al., 2001b; Moffatt et al., 2002; Kawabata, 2003a,b). Therefore, the furoylated peptides might also be useful to evaluate PAR-2 as a therapeutic target.

In conclusion, substitution of the N-terminal serine with a furoyl group in PAR-2-activating peptides dramatically enhances the agonistic activity toward PAR-2, especially in vivo, and decreases degradation by aminopeptidase, providing the most powerful PAR-2 agonist 2f-LIGRL-NH₂.

References
Kawabata A, Kawao N, Itoh H, Shimada C, nd, not determined; SMA, rat superior mesenteric artery; Ama, amastatin.


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