



Proteinase-activated receptor-2 (PAR-2): regulation of salivary and pancreatic exocrine secretion *in vivo* in rats and mice

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1 Proteinase-activated receptor-2 (PAR-2) is expressed throughout the gastrointestinal tract including the pancreas, and may be involved in digestive functions. The aim of our study was to evaluate a potential role for PAR-2 in regulating salivary and pancreatic exocrine secretion *in vivo*.

2 PAR-2-activating peptides (PAR-2-APs), but not selective PAR-1-APs, administered intravenously, increased salivary secretion in the mouse or rat; this effect of the PAR-2-APs was unaffected by atropine, phentolamine, propranolol or indomethacin.

3 Secretion (amylase) by rat parotid gland slices *in vitro* was also stimulated by PAR-2-APs and trypsin, but not by activation of other PARs.

4 PAR-2-APs, administered to rats *in vivo*, caused a prompt effect on pancreatic exocrine secretion.

5 PAR-2 mRNA, known to be present in pancreatic tissue, was also detected in parotid tissue.

6 Our results indicate that in addition to a potential role in regulating cardiovascular and respiratory functions, PAR-2 may also play a general role *in vivo* for the direct regulation of glandular exocrine secretion.

British Journal of Pharmacology (2000) **129**, 1808–1814

Keywords: Proteinase (protease)-activated receptor (PAR); salivation; parotid gland; amylase; trypsin; pancreatic juice; exocrine secretion

Abbreviations: A(Cit)p-NH₂, A(parafluoro-)FR-(cyclohexyl)-A-citrulline-Y-NH₂; FSp-NH₂, FSLLR-NH₂; GYp-OH, GYPGKF; LSp-NH₂, LSIGRL-NH₂; PAR, proteinase-activated receptor; PAR-AP, PAR-activating peptide; RT-PCR, reverse transcriptase-polymerase chain reaction; SFp-NH₂, SFLLR-NH₂; SLp-NH₂, SLIGRL-NH₂; SLp-OH, SLIGRL; tLp-NH₂, *N*-trans-cinnamoyl-LIGRL-ornithine-NH₂; TFp-NH₂, TFLLR-NH₂

Introduction

Proteinase-activated receptors (PARs) are unique members of the large superfamily of G-protein-coupled seven transmembrane receptors, the activation of which is achieved by an irreversible proteolytic mechanism (Dery *et al.*, 1998). Four distinct subtypes of PARs (PARs 1 to 4) have been described (Vu *et al.*, 1991; Nystedt *et al.*, 1994; Ishihara, H. *et al.*, 1997; Kahn *et al.*, 1998; Xu *et al.*, 1998). PAR-1, PAR-3 and PAR-4 are activated by thrombin, whereas PAR-2 is activated by trypsin or tryptase, but not by thrombin (Nystedt *et al.*, 1994; Molino *et al.*, 1997). Messenger RNAs for these four PARs are expressed in varying amounts in a wide variety of tissues in humans and other species (Vu *et al.*, 1991; Nystedt *et al.*, 1994; Ishihara *et al.*, 1997; Kahn *et al.*, 1998; Xu *et al.*, 1998; Hollenberg, 1999). Remarkably, except for PAR-3, synthetic receptor-activating peptides (PAR-APs) as short as 5–6 amino acids, based on the N-terminal receptor-activating motifs of the tethered ligands, are capable of activating the receptors directly by a mechanism independent of proteolysis (e.g. the peptide, SFLLR for human PAR-1, SLIGRL for mouse and rat PAR-2 and GYPGKF for mouse PAR-4) (Vu *et al.*, 1991; Nystedt *et al.*, 1994; Ishihara *et al.*, 1997; Kahn *et al.*, 1998; Xu *et al.*, 1998). Studies of the peptide structure-activity relationships using a variety of PAR-AP analogues have led to the

development of receptor-selective agonists for PAR-1 and PAR-2 (Hollenberg *et al.*, 1997; Kawabata *et al.*, 1999b; Blackhart *et al.*, 1996). As yet, receptor-selective antagonists for the PARs have not been developed.

Although a haemostatic/cardiovascular role for PARs-1, 3 and 4 can be suggested because of the activation of human and murine platelets by thrombin (Dery *et al.*, 1998; Vu *et al.*, 1991; Kahn *et al.*, 1998; 1999), the physiological or pathophysiological role(s) for PAR-2 are still essentially unknown. It has been our working hypothesis that PAR-2, like PAR-1, may be involved in inflammatory or injury-response events (Cirino *et al.*, 1996; Kawabata *et al.*, 1998; 1999a; Vergnolle *et al.*, 1999). PAR-2 activation is known to cause the contraction or relaxation of vascular, gastric and airway smooth muscle (Hollenberg *et al.*, 1997; Saifeddine *et al.*, 1996; Cocks *et al.*, 1999). Further, activation of PAR-2 *in vivo* has been observed to cause hypotension in mice and other mammals (Cheun *et al.*, 1998; Damiano *et al.*, 1999) and to inhibit bronchoconstriction in rats (Cocks *et al.*, 1999). Amongst other tissues, the digestive tract and pancreas have been found to express high amounts of PAR-2 mRNA (Bohm *et al.*, 1996). In isolated tissue preparations, PAR-2-APs and trypsin have been found to modulate intestinal function (Kon *et al.*, 1997; Corvera *et al.*, 1997; Vergnolle *et al.*, 1998); and PAR-2-APs have also been observed to cause amylase secretion from isolated pancreatic acinar fragments (Bohm *et al.*, 1996). Most recently, a study of tissue *in vitro* has demonstrated that canine pancreatic duct cells are rich in PAR-2 and that trypsin

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transiently activates pancreatic duct epithelial cell ion channels by triggering PAR-2 (Nguyen *et al.*, 1999).

Given the high levels of PAR-2 mRNA in the digestive tract (Hollenberg, 1999) and the demonstrated effects of PAR-2 activation on isolated pancreatic tissue *in vitro* (Bohm *et al.*, 1996; Nguyen *et al.*, 1999), we hypothesized that PAR-2 might play a more general role in regulating glandular exocrine function, related to the digestive process. We therefore turned our attention to the parotid gland, which plays an auxiliary digestive role. This tissue is readily accessible for studies both *in vivo* and *in vitro*. We assessed the activities of PAR-2-APs on parotid secretion in mice and rats *in vivo* and on rat parotid slice secretion *in vitro*. Further, we examined whether PAR-2 activation *in vivo* might trigger rat pancreatic secretion. Our data indicated that PAR-2 (but not PAR-1) may play an important role in regulating salivary and pancreatic secretion *in vivo*.

Methods

Experimental animals

Male ddY mice weighing 20–30 g and Wistar rats weighing 200–300 g (Japan SLC, Inc.) were used with approval from the Kinki University Faculty of Pharmaceutical Sciences' Committee for the care and use of laboratory animals. Mice were employed for the detailed *in vivo* studies (dose-response curves; inhibitors) to economize the amount of peptides.

In vivo salivation bioassay in the mouse and rat

Rats and mice were anaesthetized with i.p. urethane (1.5 g kg⁻¹) and the salivation bioassay was performed essentially according to the previously described method with modifications (Takeda *et al.*, 1989). Immediately before drug challenge, saliva in the animal's oral cavity was removed using an aspirator. Then whole saliva was successively aspirated at 1 min intervals for 5 min after i.v. administration of secretagogues. The amount of saliva collected during each 1 min interval was quantitated by weight (mg).

Drug administration schedules in the *in vivo* salivation assay

The specific PAR-2-APs, SLIGRL-NH₂ (SLp-NH₂), SLIGRL (SLp-OH) and *N*-trans-cinnamoyl-LIGRL-ornithine-NH₂ (tcLp-NH₂), or their inactive control peptide LSIGRL-NH₂ (LSp-NH₂), the PAR-1/PAR-2 agonist SFLLR-NH₂ (SFp-NH₂) or its inactive control peptide FSLLR-NH₂ (FSp-NH₂), and the specific PAR-1-AP TFLLR-NH₂ (TFp-NH₂), in a dose range of 0.5–50 µmol kg⁻¹, and carbachol at 0.08 µmol kg⁻¹ were administered i.v. to the mouse or rat. Amastatin at 2.5 or 84 µmol kg⁻¹ was preadministered i.v. 1 min before i.v. injection of SLp-NH₂ at 0.5 µmol kg⁻¹. Atropine at 7.2 µmol kg⁻¹ (5 mg kg⁻¹), phentolamine at 15.7 µmol kg⁻¹ (5 mg kg⁻¹), propranolol at 16.9 µmol kg⁻¹ (5 mg kg⁻¹) or indomethacin at 28 µmol kg⁻¹ (10 mg kg⁻¹) were administered i.p. 25 min before i.v. SLp-NH₂ at 5 µmol kg⁻¹. Control animals received i.v. or i.p. injection of vehicle.

Determination of composition of saliva secreted *in vivo*

In the mouse, the saliva secreted in response to i.v. SLp-NH₂ at 5 µmol kg⁻¹ or carbachol at 0.08 µmol kg⁻¹ was collected and subjected to compositional analysis. Amylase activity in the

collected saliva was measured spectrophotometrically using an assay kit (Amylase B-test, Wako, Japan). Sodium and potassium concentrations were determined by an automatic electrolyte analyser with ion-selective electrodes (664, Chiron, U.S.A.).

Detection of mRNA for PAR-1 and PAR-2 in rat parotid gland and pancreas by a reverse transcriptase-polymerase chain reaction

Total RNA was isolated from rat parotid gland and from rat pancreas as a positive control, using the TRIzol Reagent (Life Technologies, Inc., U.S.A.), and then mRNA was purified from the total RNA with the Oligotex-dT30 (Super) mRNA purification kit (Takara Shuzo, Japan). Essentially as described previously (Hollenberg *et al.*, 1996), mRNA was reverse-transcribed at 42°C for 50 min, and amplified by polymerase chain reaction (RT-PCR) using the RNA LA PCR kit (AMV) ver. 1.1 (Takara Shuzo, Japan). The PCR primers for amplification of PAR-1 sequences were 5'-CCCGCTCATTTTTCTCAGGA-3' and 5'-GCCAATCG-GTCGCGGAGAAGT-3', leading to amplification of 394 bp fragments. The primers targeted to PAR-2 were 5'-CACCAG-TAAAGGGAGAAGTCT-3' and 5'-GGCAGCACGTCGT-GACAGGT-3', yielding amplification of 598 bp fragments. The primers for β-actin were 5'-GTGGGGCGCCCCAGG-CACCA-3' and 5'-GTCCTTAATGTCACGCACGATTTC-3', amplifying 537-bp fragments. Amplification was allowed to proceed for 35 cycles beginning with a 30 s denaturation period at 94°C followed by a 30 s reannealing time at 55°C and a 1 min primer extension period at 72°C. The PCR products were separated by 2% agarose gel electrophoresis and visualized by the ethidium bromide staining procedure.

In vitro assay of amylase secretion from rat parotid gland slices

Essentially according to the previously described method (Jahn *et al.*, 1980), the rat parotid glands were removed under sodium pentobarbital (50 mg kg⁻¹, i.p.) anaesthesia, and immediately washed with an ice-cold, gassed (95% O₂ plus 5% CO₂) Krebs–Henseleit buffer of the following composition (mM): NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgCl₂, NaHCO₃, 25; KH₂PO₄, 1.2; glucose 10. Glands were quickly freed from adherent fat and connective tissue and cut into small pieces of about 1 mm³ in the iced buffer under a dissecting microscope. Portions of 100 mg wet weight of the slices were preincubated for 30 min in 3 ml of the same buffer that was continuously gassed with 95% O₂/5% CO₂ and maintained at 37°C, and a small aliquot of 30 µl was collected (background sample, BS). Then the sample solution was incubated for 10 min after addition of PAR-APs, trypsin or thrombin at various concentrations; at that time another 30 µl aliquot was collected (reaction sample, RS). Thereafter, the slices in the buffer solution were homogenized and centrifuged at 30,000 *g* and at 4°C for 30 min. The supernatant was used to determine the total amount of amylase present in the assay system (cells plus buffer) (total sample, TS). Amylase activity in BS, RS and TS was measured as described above, and amylase secretion (%) was calculated as follows: (RS-BS)/(TS-BS) × 100.

In vivo bioassay of secretion of pancreatic juice in the rat

Pancreatic exocrine secretion in the rat was assessed as described previously (Tachibana *et al.*, 1996). Briefly, under

urethane anaesthesia, the common bile-pancreatic duct of the rat was cannulated at the distal end, and then the bile duct was ligated at a distal part above the common bile-pancreatic duct and cannulated at a proximal part to the liver above the ligature. Bile was diverted *via* a bypass into the duodenum, and the pure pancreatic juice was collected continuously from the end of the cannula connected to the common bile-pancreatic duct using a capillary and weighed every 5 min for quantitation. After a 30 min stabilization period, the PAR-2-AP, SLp-NH₂ or the control peptide LSp-NH₂ at 5 $\mu\text{mol kg}^{-1}$ in combination with amastatin at 2.5 $\mu\text{mol kg}^{-1}$ were administered *i.v.* to the rat.

Peptides and other reagents

All peptides used were prepared by solid-phase synthesis either by the Peptide Synthesis Core Facility, Department of Medical Biochemistry, University of Calgary (Canada), or by ourselves. Peptide composition and purity (>98%) were ascertained by HPLC analysis, amino acid analysis and mass spectrometry. Carbachol, phentolamine hydrochloride, trypsin from porcine pancreas and human thrombin were purchased from Sigma (U.S.A.), atropine sulphate, indomethacin and tetrodotoxin were obtained from Wako (Japan), amastatin and spantide were supplied from Peptide Institute (Japan), and propranolol hydrochloride was from Tokyo Kasei (Japan).

Data analysis

Results are expressed as means \pm s.e.m. Statistical significance of differences between groups was analysed by Student's *t*-test or Tukey's multiple comparison test, and $P < 0.05$ was accepted as significant.

Results

In vivo salivation triggered by PAR-2-APs in the mouse

SFLLR-NH₂ (SFp-NH₂), a thrombin/PAR-1 agonist used previously by others, that can also activate PAR-2 (Kawabata *et al.*, 1999b), when administered *i.v.* at a dose of

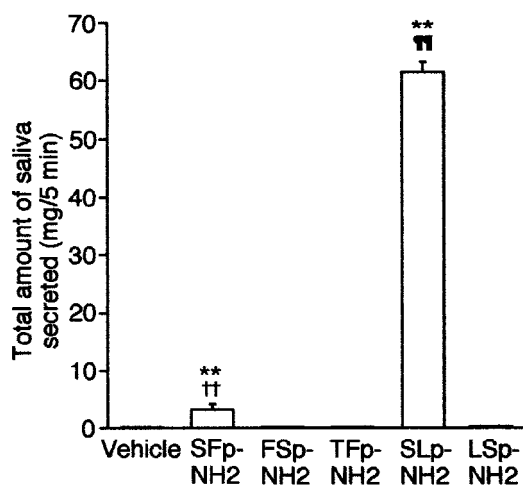


Figure 1 Effects of systemic (*i.v.*) administration of PAR-1-APs and PAR-2-APs on *in vivo* salivation in the mouse. The saliva secreted was collected for 5 min after *i.v.* administration of each peptide at 15 $\mu\text{mol kg}^{-1}$. Data indicate the mean with s.e.mean from 4 mice. ** $P < 0.01$ vs vehicle; †† $P < 0.01$ vs FSp-NH₂; ††† $P < 0.01$ vs LSp-NH₂.

15 $\mu\text{mol kg}^{-1}$, evoked secretion of a small amount of saliva for 5 min in the mouse, while the inactive control peptide, FSLLR-NH₂ (FSp-NH₂), at the same dose had no effect. TFLLR-NH₂ (TFp-NH₂, 15 $\mu\text{mol kg}^{-1}$), a highly selective PAR-1-AP that is equipotent with SFp-NH₂ for activating PAR-1 (Hollenberg *et al.*, 1997), given in the same manner, failed to trigger any salivation. In contrast, SLIGRL-NH₂ (SLp-NH₂), a selective PAR-2-AP, administered *i.v.* at 15 $\mu\text{mol kg}^{-1}$, produced a marked salivation, although the inactive control peptide LSLIGRL-NH₂ (LSp-NH₂) had no effect (Figure 1).

In the time-course experiments, *i.v.* administration of SLp-NH₂ at 5 $\mu\text{mol kg}^{-1}$ rapidly evoked salivation, the effect peaking after 1 min and disappearing after 3 min, although vehicle (saline) or LSp-NH₂ at the same dose, given *i.v.*, did not induce any saliva secretion responses throughout 5 min (Figure 2A). The effect of SLp-NH₂ was dose-dependent in a range of 0.5–5 $\mu\text{mol kg}^{-1}$. SLp-NH₂ at the largest dose tested, 50 $\mu\text{mol kg}^{-1}$, was less effective than that at 5 $\mu\text{mol kg}^{-1}$. The dose-response curve for the effect of this compound thus exhibited a bell shape. The control peptide LSp-NH₂ remained ineffective throughout the same dose range. SLIGRL (SLp-OH), administered *i.v.*, also evoked salivation in a dose-dependent manner, but its effective dose range was high (5–50 $\mu\text{mol kg}^{-1}$), relative to the potency of SLp-NH₂, in agreement with the previous studies demonstrating a higher potency of SLp-NH₂ relative to SLp-OH for activating PAR-2 *in vitro* and *in vivo* (Kawabata *et al.*, 1998; Saifeddine *et al.*, 1996) (Figure 2B). Another PAR-2-AP derivative, *N*-trans-cinnamoyl-LIGRL-ornithine-NH₂ (tcLp-NH₂) at 0.5–1.5 $\mu\text{mol kg}^{-1}$ caused salivation equivalent to that caused by the same dose of SLp-NH₂. In this assay, tcLp-NH₂ appeared to be a 'partial agonist', since tcLp-NH₂ at doses higher than 5 $\mu\text{mol kg}^{-1}$ was unable to give the maximum effect (Figure 2B). Amastatin, an inhibitor of aminopeptidase that is a major enzyme responsible for degradation of PAR-related peptides (Saifeddine *et al.*, 1996; Hollenberg *et al.*, 1993), when preadministered *i.v.* at 2.5 or 84 $\mu\text{mol kg}^{-1}$, dramatically enhanced the salivation response to a dose of SLp-NH₂ (0.5 $\mu\text{mol kg}^{-1}$) that was ineffective by itself, although amastatin at those doses alone was incapable of inducing salivation. Amastatin at the two distinct doses was almost equipotent in terms of facilitation of the effect of SLp-NH₂, indicating that the effect of amastatin at 2.5 $\mu\text{mol kg}^{-1}$ was already maximal (Figure 2C).

Atropine (7.2 $\mu\text{mol kg}^{-1}$), phentolamine (15.7 $\mu\text{mol kg}^{-1}$), propranolol (16.9 $\mu\text{mol kg}^{-1}$) and indomethacin (28 $\mu\text{mol kg}^{-1}$), preadministered *i.p.*, failed to attenuate or enhance salivation evoked by *i.v.* SLp-NH₂ at 5 $\mu\text{mol kg}^{-1}$; the amount of saliva secreted (mg per 5 min) in the mice pretreated with vehicle, atropine, phentolamine, propranolol and indomethacin was 48.8 ± 4.3 , 57.6 ± 6.8 , 54.4 ± 6.2 , 66.5 ± 10.0 and 50.5 ± 8.9 ($n = 4$), respectively.

Comparison of composition of the saliva secreted in response to the PAR-2-AP, SLp-NH₂, and by carbachol in the mouse

To characterize salivation induced by activation of PAR-2 in the mouse, the composition of the saliva (total amount: 37.4 ± 1.4 mg per 5 min, $n = 4$) secreted in response to *i.v.* SLp-NH₂ at 5 $\mu\text{mol kg}^{-1}$ was compared to saliva secreted in response to *i.v.* carbachol at a dose (0.08 $\mu\text{mol kg}^{-1}$) that induced an equivalent amount of saliva (total amount: 41.4 ± 4.3 mg per 5 min, $n = 4$). The concentrations of Na⁺ and K⁺ ions (95.0 ± 15.1 and 72.5 ± 4 , mEq L⁻¹), and the

activity of amylase ($981.8 \pm 35.5 \times 10^3$ IU L⁻¹) in the saliva secreted due to SLp-NH₂ were comparable to those due to carbachol (96.3 ± 3.1 and 92.5 ± 5.2 mEq L⁻¹; $723.8 \pm 122.6 \times 10^3$ IU L⁻¹), respectively, although a minor significant ($P < 0.05$) difference was seen in the K⁺ ion levels, being consistent with the previous hypothesis that PAR-2, like the muscarinic receptor, is likely coupled to G_q thereby stimulating phospholipase C in some tissues or cells (Dery *et al.*, 1998).

PAR-2-APs-induced parotid gland secretion in vivo and in vitro in the rat, and RT-PCR detection of PAR-2 mRNA

In the rat, as observed in the mouse, the specific PAR-2-AP, SLp-NH₂, but not its inactive control LSp-NH₂ or the

specific PAR-1-AP, TFp-NH₂, when administered *in vivo* ($5 \mu\text{mol kg}^{-1}$), triggered significant salivation, with an effect peaking at 1 min and lasting up to 4 min (Figure 3A).

The selective PAR-2-APs, SLp-NH₂ and tcLp-NH₂ at 10–100 μM evoked secretion of amylase from rat parotid slices *in vitro*, in a concentration-dependent manner, while LSp-NH₂ at 100 μM had no effect. The PAR-2-activating enzyme trypsin at 0.13 μM , also induced significant secretion of amylase from the slices, although a larger concentration, 0.26 μM , of trypsin produced a smaller effect. The selective PAR-1-APs, TFp-NH₂ or A(parafluoro-)FR-(cyclohexyl)-A-citrulline-Y-NH₂ [A(Cit)p-NH₂] at 100 μM , the PAR-4-AP, GYPGKF (GYp-OH) at 500 μM and thrombin at 0.1–0.2 μM did not trigger amylase secretion from the parotid slices (Figure 3C).

In the rat pancreas, used as the positive control, mRNAs for PAR-1 and for PAR-2 were readily detected by RT-PCR (Figure 3B, left, lanes P-1 and P-2). The RT-PCR analysis of mRNA harvested from the rat parotid gland also yielded PCR products of the predicted size, 394 bp for PAR-1 and 598 bp for PAR-2, respectively (Figure 3B, right, lanes P-1 and P-2), indicating expression of both PAR-1 and PAR-2 in the rat parotid gland.

In vivo pancreatic exocrine secretion responses to PAR-2 activation by SLp-NH₂ in the rat

Finally, as a comparative experiment, we examined if the administration of PAR-2-APs *in vivo* would, as for the parotid gland, also trigger pancreatic exocrine secretion. The PAR-2-AP, SLp-NH₂ at $5 \mu\text{mol kg}^{-1}$, when co-administered *i.v.* with amastatin at $2.5 \mu\text{mol kg}^{-1}$, produced a prompt increase in pancreatic juice secretion for 0–5 min after the administration, followed by a pronounced and transient suppression of pancreatic secretion for 5–10 min, with a subsequent long-lasting increase in secretion that lasted for 15–60 min (Figure 4).

Discussion

Although discovered in 1994 (Nystedt *et al.*) by reduced stringency cloning from a murine cDNA library, the physiological and/or pathophysiological role that PAR-2 may play *in vivo* has yet to be defined. The main finding of our study was that the activation of PAR-2 *in vivo*, with the use of receptor-selective activating peptides, led to both salivary and pancreatic secretion. Data obtained with the parotid slice preparation *in vitro* paralleled exactly the results obtained *in vivo*. Our work thus adds the regulation of exocrine glandular secretion to the list of possible roles that the novel PAR-2 system might subserve *in vivo*. Our data obtained in intact animals are in complete accord with results obtained with isolated pancreatic acini and cultured pancreatic duct epithelial cells *in vitro* (Bohm *et al.*, 1996; Nguyen *et al.*, 1999); but our data obtained *in vivo* suggest a more complex regulation of pancreatic secretion by PAR-2 (multi-phasic response) than was suggested by the studies done *in vitro* (Bohm *et al.*, 1996; Nguyen *et al.*, 1999). Comparable studies done *in vivo* with PAR-2-activating reagents have also pointed to a role for PAR-2 in the regulation of the cardiovascular (Cheun *et al.*, 1998; Damiano *et al.*, 1999) and respiratory (Cocks *et al.*, 1999) functions.

It is of note that the highly selective PAR-1-AP, TFp-NH₂, did not cause salivary secretion *in vivo*, and that neither thrombin nor the selective PAR-1-APs [TFp-NH₂ and A(Cit)p-NH₂] and PAR-4-AP (GYp-NH₂) caused amylase release from rat parotid slices *in vitro* (Figure 3C). In contrast,

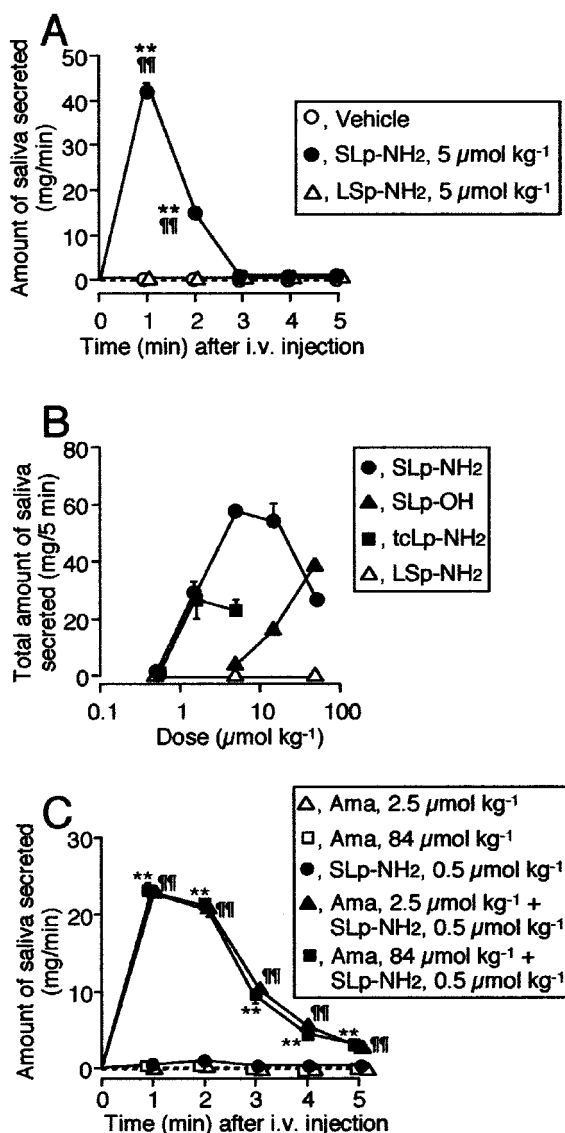


Figure 2 Salivation evoked by PAR-2-APs, administered *i.v.*, in the mouse. (A) Time course to *i.v.* SLp-NH₂ or LSp-NH₂ at $5 \mu\text{mol kg}^{-1}$. The amount of saliva secreted was quantitated every minute for 5 min. (B) Dose-related effects of *i.v.* SLp-NH₂, SLp-OH, tcLp-NH₂ or LSp-NH₂ on saliva secretion for 5 min. (C) Potentiation by amastatin of SLp-NH₂-induced salivation. Amastatin (Ama) at 2.5 or 84 $\mu\text{mol kg}^{-1}$ was administered *i.v.* 1 min before *i.v.* SLp-NH₂ at $0.5 \mu\text{mol kg}^{-1}$. Data indicate the mean with s.e.mean from four mice. ** $P < 0.01$ vs vehicle, and ¶ $P < 0.01$ vs LSp-NH₂ in (A); ** $P < 0.01$ vs Ama at 84 $\mu\text{mol kg}^{-1}$, and ¶ $P < 0.01$ vs Ama at 2.5 $\mu\text{mol kg}^{-1}$ in (C).

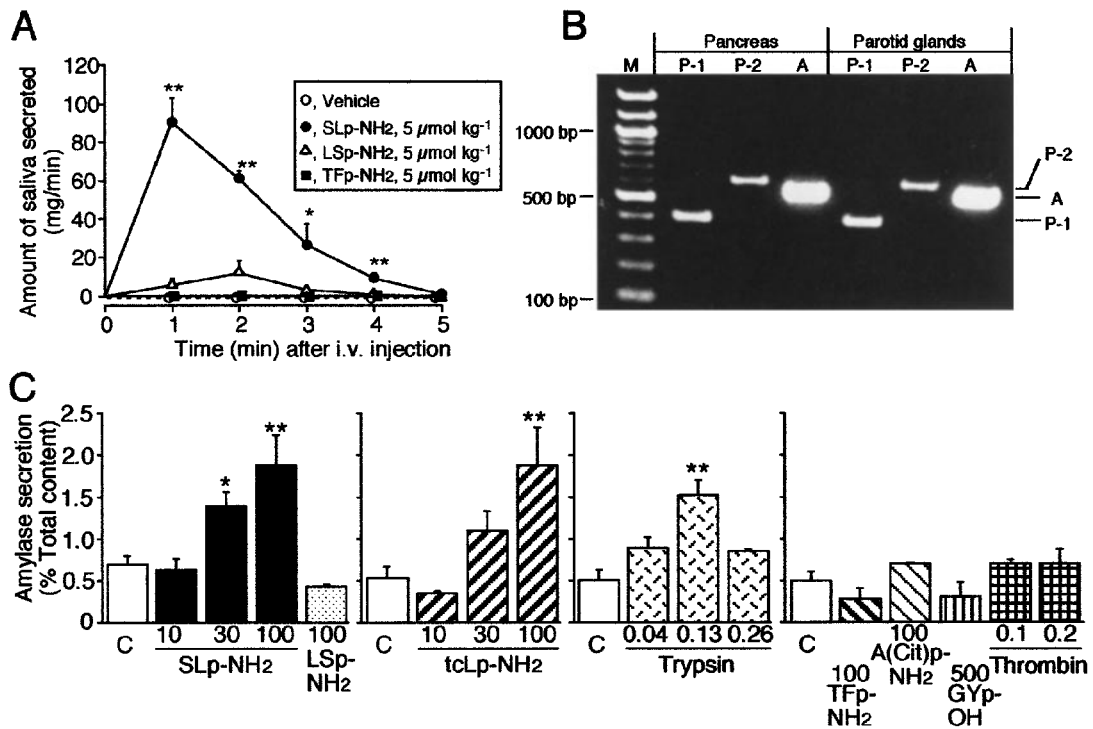


Figure 3 PAR-2-mediated *in vivo* and *in vitro* salivation in the rat, and RT-PCR detection of PAR-2 mRNA in the rat salivary gland. (A) *In vivo* salivation induced by PAR-2 activation in the rat. SLp-NH₂, LSp-NH₂ and TFP-NH₂ were administered *i.v.* to the rat. The amount of saliva secreted was quantitated every minute for 5 min. Data indicate the mean with s.e.mean from four rats. **P* < 0.05, ***P* < 0.01 vs vehicle. (B) RT-PCR analysis of mRNA harvested from the rat parotid gland and from the rat pancreas for detection of PAR-2. Primers were designed to PAR-1 (lane P-1), PAR-2 (lane P-2) and actin (lane A). The positions of the predicted PCR products are shown on the right. M, marker. (C) *In vitro* secretion of amylase from the rat parotid slices. The slices were incubated at 37°C for 10 min, with peptides or enzymes at various concentrations (μ M). Data indicate the mean with s.e.mean from nine control (C) and 4–6 test experiments. **P* < 0.05, ***P* < 0.01 vs the control.

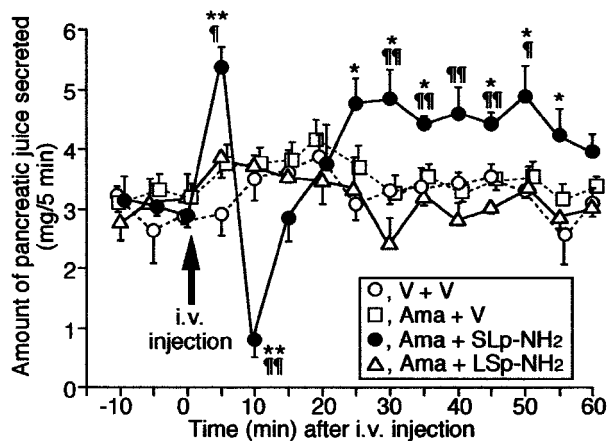


Figure 4 Effect of the PAR-2-AP, SLp-NH₂, administered *i.v.*, on *in vivo* pancreatic juice secretion in the rat. SLp-NH₂ or LSp-NH₂ at 5 μ mol kg⁻¹, in combination with amastatin at 2.5 μ mol kg⁻¹, were administered *i.v.* to the rat. The amount of the juice was quantitated every 5 min. V: Vehicle. Data indicate the mean with s.e.mean from four rats. **P* < 0.05, ***P* < 0.01 vs V+V; ¶*P* < 0.05, ¶¶*P* < 0.01 vs Ama+LSp-NH₂.

the PAR-2-selective activating peptides, SLp-NH₂ and tLp-NH₂, were equipotent in this regard, whereas the partial-reverse-sequence PAR-2-derived peptide, LSp-NH₂, known not to activate PAR-2, was inactive. It is recognized that SLp-NH₂ is unable to activate PAR-1, PAR-3 or PAR-4, since this peptide cannot, as do either thrombin or the PAR-1- and PAR-4-APs, cause aggregation of human (PAR-1 plus PAR-4)

or murine (PAR-3 plus PAR-4) platelets (Vu *et al.*, 1991; Ishihara *et al.*, 1997; Kahn *et al.*, 1998; 1999). Further, our salivary secretion data, showing that SLp-NH₂ and tLp-NH₂ were equipotent *in vivo* as well as *in vitro*, support the conclusion that the effects were due to PAR-2 itself, and not to other pharmacologically distinct receptors that can also be activated by SLp-NH₂ (Vergnolle *et al.*, 1998; Roy *et al.*, 1998; Saifeddine *et al.*, 1998). The low, but detectable activity in the salivation assay of one of the originally-described thrombin-receptor (PAR-1) activating peptides [SFp-NH₂ (formerly designated TRAP); Figure 1] can be understood in terms of its ability to activate PAR-2 (Kawabata *et al.*, 1999b). The ball-shaped dose-response curve for the salivation effect of SLp-NH₂ *in vivo* (Figure 2B) might suggest the presence of unknown negative feedback systems or the non-specific actions of this peptide at large doses, although the detailed mechanisms remain to be investigated. The pharmacological profile for the salivary secretion assay, *in vivo* as well as *in vitro* [trypsin >> SLp-NH₂ = tLp-NH₂; LSp-NH₂, A(Cit)p-NH₂ and TFP-NH₂ inactive] parallels exactly the structure-activity profile for the agonists in stimulating calcium signalling in rat PAR-2-expressing KNRK cells (Vergnolle *et al.*, 1998). Further, the range of concentration over which trypsin caused amylase secretion from parotid slices *in vitro* was comparable to the concentrations of trypsin that cause PAR-2-mediated intestinal prostaglandin secretion (Kong *et al.*, 1997), canine pancreatic duct epithelial cell ion channel activation (Nguyen *et al.*, 1999) and the suppression of colonic motility (Corvera *et al.*, 1997). Our data thus strongly support a role for the trypsin/tryptase-activated PAR-2 in regulating salivary secretion, rather than the thrombin-activated receptors, PARs-1,3 or 4.

Given the unusual proteinase-mediated mechanism for the activation of PAR-2 and an absence of a conventional circulating 'hormonal' ligand, an interesting question to pose is: under what circumstances might this receptor become activated, so as to subserve a physiological/pathophysiological role? Our working hypothesis has been that the PARs in general play roles in the setting of an inflammatory response, injury or during organ development (Hollenberg *et al.*, 1999). In this context, it can be pointed out that exposure of rats to an intestinal antigenic challenge can result in the release of mast cell proteinases into the circulation (Scudamore *et al.*, 1995) and that elevated levels of α -tryptase can be detected in the blood of individuals with systemic mastocytosis (Schwartz *et al.*, 1995). Our data showing that PAR-2 activation stimulates pancreatic exocrine secretion would imply that the sensitivity of pancreatic PAR-2 to circulating α -tryptase might account for the increased incidence of peptic ulcer disease in individuals with systemic mastocytosis. PAR-2 activation might also

account for other symptomatology in such individuals (diarrhoea, hypotension and urticaria), an area that merits further study. Whether circulating mast cell tryptases (human) or chymases (rat) may account for a stimulation of exocrine glandular secretion remains an interesting topic for further exploration. That our data imply a general effect of PAR-2 activation on exocrine glandular secretion suggests that potential of generalized systemic effect that could be brought about by the release (due to inflammation or other causes) into the circulation of PAR-2-targeted proteinases. Taken together with its other roles in gastrointestinal tract (Hollenberg *et al.*, 1997; Saifeddine *et al.*, 1996; Kong *et al.*, 1997; Corvera *et al.*, 1997), PAR-2 appears to play a key role in the digestive systems, and may provide a novel scope for drug development.

The work described here was supported in part by research grant No. 11672283 from the Japanese Ministry of Education, Science and Culture.

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(Received November 5, 1999
Revised January 27, 2000
Accepted February 2, 2000)