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In vivo evidence that protease-activated receptors 1 and 2 modulate gastrointestinal transit in the mouse

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1 Protease-activated receptors (PARs) 1 and 2 modulate the gastric and intestinal smooth muscle motility *in vitro*. In the present study, we examined if activation of PAR-2 and PAR-1 could alter gastrointestinal transit in mice.

2 Intraperitoneal administration of the PAR-2-activating peptide SLIGRL-NH_2, but not the inactive control LSIGRL-NH_2, at 1–5 μmol kg^{-1}, in combination with the aminopeptidase inhibitor amastatin at 2.5 μmol kg^{-1}, facilitated gastrointestinal transit in a dose-dependent manner. The human PAR-1-derived peptide SFLLR-NH_2 and the specific PAR-1 agonist TFLLR-NH_2, but not the inactive control FSLLR-NH_2, at 2.5–10 μmol kg^{-1}, in combination with amastatin, also promoted gastrointestinal transit.

3 The Ca^{2+}-activated, small conductance K^+ channel inhibitor apamin at 0.01 μmol kg^{-1} significantly potentiated the actions of SLIGRL-NH_2 and TFLLR-NH_2 at subeffective doses.

4 The increased gastrointestinal transit exerted by either SLIGRL-NH_2 at 5 μmol kg^{-1} or TFLLR-NH_2 at 10 μmol kg^{-1} was completely abolished by the L-type Ca^{2+} channel inhibitor verapamil at 61.6 μmol kg^{-1}. In contrast, the tyrosine kinase inhibitor genistein at 18.5 μmol kg^{-1} failed to modify the effects of the agonists for PAR-2 or PAR-1.

5 These findings demonstrate that PAR-1 and PAR-2 modulate gastrointestinal transit in mice *in vivo*. Our data also suggest that the PAR-1-and PAR-2-mediated effects are modulated by apamin-sensitive K^+ channels and are dependent on activation of L-type Ca^{2+} channels, but independent of tyrosine kinase. Our study thus provides novel evidence for the physiological and/or pathophysiological roles of PARs 1 and 2 in the digestive systems, most probably during inflammation.

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Abbreviations: PAR, protease-activated receptor

Introduction

Thrombin exhibits cellular actions mainly by activating protease-activated receptors (PARs) 1, 3 or 4 (Vu *et al*., 1991; Ishihara *et al*., 1997; Kahn *et al*., 1998; Xu *et al*., 1998; Nakanishi-Matsui *et al*., 2000), while trypsin and mast cell tryptase, do so through activation of PAR-2 (Nystedt *et al*., 1994; Molino *et al*., 1997). Activation of PARs is achieved by proteolytic unmasking of the cryptic tethered ligand present in the N-terminal peptide of the receptor (for review, see Dery *et al*., 1998; Hollenberg, 1999; Kawabata & Kuroda, 2000). Amongst the four members of the PAR family, PARs 1 and 2 are now recognized as physiologically important molecules, especially in the early stages of inflammation, tissue-injury or haemorrhage, with protective and/or aggravating effects (Cirino *et al*., 1996; Kawabata *et al*., 1998; 1999a; Hollenberg, 1999; Vergnolle *et al*., 1999a, b; Kawabata & Kuroda, 2000; Cocks & Moffatt, 2000).

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The alimentary systems are, in particular, abundant in PARs 1 and 2 (Hollenberg, 1999; Kawabata & Kuroda, 2000). PAR-2 or PAR-2-like receptors are involved in salivary and pancreatic exocrine secretion (Bohm *et al*., 1996; Nguyen *et al*., 1999; Kawabata *et al*., 2000c, d), and in intestinal ion transport (Vergnolle *et al*., 1998). There is also *in vitro* evidence that PARs 1, 2 and 4 modulate smooth muscle motility throughout the gastrointestinal tract including the oesophagus (Hollenberg *et al*., 1993; 1997; 1999; Corvera *et al*., 1997; Zheng *et al*., 1998; Cocks *et al*., 1999; Kawabata *et al*., 1999b; 2000a, b). Agonists for PARs 1, 2 and 4 produce contraction of the gastric longitudinal smooth muscle from rats or guinea-pigs (Hollenberg *et al*., 1993; 1997; 1999; Saifeddine *et al*., 1996; Zheng *et al*., 1998), whereas activation of PARs 1 and 2 elicits relaxation of the precontracted smooth muscle of mouse gastric fundus or guinea-pig taenia coli (Cocks *et al*., 1999). Further, PAR-1 agonists exhibit a dual action, relaxation followed by contraction, in the rat duodenal smooth muscle, while PAR-2 agonists elicit only contraction and PAR-4 agonists

have no effect in the same tissue (Kawabata *et al*., 1999b; 2000b). The signal transduction mechanisms for the contraction and/or relaxation of smooth muscle due to activation of PARs may include activation of L-type Ca^{2+} channels, tyrosine kinase and apamin-sensitive K^+ channels in some tissues, but do not necessarily appear to be common in distinct tissues (Hollenberg *et al*., 1993; 1997; 1999; Corvera *et al*., 1997; Zheng *et al*., 1998; Cocks *et al*., 1999; Kawabata *et al*., 1999b; 2000a, b). Questions that have yet to be answered are: (1) what happens *in vivo* as an outcome of activation of PAR-1 and PAR-2 in the gastrointestinal tract? and (2) can the mechanisms suggested in previous *in vitro* studies as described above be demonstrated *in vivo*? In the present study, therefore, we examined if agonists for PAR-1 and PAR-2 could modulate gastrointestinal transit in the mouse *in vivo*, and attempted to characterize the mechanisms of the *in vivo* modulation of gastrointestinal transit through PARs.

Methods

Animals employed

Male ddY mice weighing 25–35 g (Japan SLC. Inc., Japan) were used with approval from the Kinki University Faculty of Pharmaceutical Sciences' Committee for the Care and Use of Laboratory Animals. After 1 week of acclimatization (temperature $24\pm1°C$; humidity 60%), food was withheld for 16–20 h before experiments, but animals had free access to tap water.

Assessment of gastrointestinal transit

As described elsewhere (Izzo *et al*., 2000), 10% charcoal suspension in 5% gum arabic was administered orally to conscious mice, and the mice were killed by cervical dislocation after 20 min. The gastrointestinal tract was removed, and the length of the small intestine traversed by the marker was measured. Data are expressed as percentages of the total length of the small intestine of each mouse.

Drug administration schedules

PAR-related peptides used were: the PAR-2 agonist SLIGRL-NH_2 (based on murine and rat PAR-2), the PAR-2-inactive control peptide LSIGRL-NH_2, the PAR-1 agonist SFLLR-NH_2 (based on human PAR-1) and TFLLR-NH_2, and the PAR-1-inactive control peptide FSLLR-NH_2. These peptides at various doses were administered intraperitoneally (i.p.) to the mouse 1 min after i.p. amastatin at 2.5 μmol kg^{-1} (Kawabata *et al*., 2000d), and followed immediately by the charcoal meal administered orally. In some experiments, carbachol at 0.055 or 0.55 μmol kg^{-1} was administered i.p. without amastatin. The Ca^{2+}-dependent, small conductance K^+ channel inhibitor apamin at 0.01 μmol kg^{-1} was given i.p. 6 min before i.p. administration of the agonist peptides, SLIGRL-NH_2 at 1 μmol kg^{-1} or TFLLR-NH_2 at 2.5 μmol kg^{-1} (5 min before amastatin); the dose of apamin was decided on the basis of our previous *in vitro* study where apamin at 0.1 μM completely abolished the relaxation of duodenal smooth muscle in response to TFLLR-NH_2 at 50 μM (Kawabata *et al*., 1999b). The L-type Ca^{2+} channel inhibitor verapamil at 61 μmol kg^{-1} (30 mg kg^{-1}) (Rupniak *et al*., 1993) and the tyrosine kinase inhibitor genistein at 18.5 μmol kg^{-1} (5 mg kg^{-1}) (Campos *et al*., 1999; Deodato *et al*., 1999) were administered 16 min before SLIGRL-NH_2 at 5 μmol kg^{-1} or TFLLR-NH_2 at 10 μmol kg^{-1} (15 min before amastatin). In control experiments, each vehicle was administered in the same manner.

Drugs employed

All peptides were prepared by a standard solid-phase synthesis method by ourselves. The concentration, purity and composition of the peptides were determined by high-performance liquid chromatography, mass spectrometry and quantitative amino acid analysis. Amastatin was purchased from Peptide Institute Inc. (Japan), and genistein, apamin, verapamil hydrochloride and carbachol were from Sigma (U.S.A.). Genistein was dissolved in 5% Tween 80 solution. All other chemicals were dissolved in phosphate-buffered saline or saline.

Statistical analysis

The results are represented as mean$\pm$s.e.mean. Statistical significance was analysed by Newman-Keuls' multiple comparison test, and was set at a $P<0.05$ level.

Results

Effects of receptor-activating peptides for PAR-2 and PAR-1 on gastrointestinal transit in mice

The murine PAR-2-derived receptor-activating peptide SLIGRL-NH_2 at 1–5 μmol kg^{-1}, when administered i.p. in combination with i.p. amastatin, an inhibitor of aminopeptidase, a peptide degrading enzyme, at 2.5 μmol kg^{-1}, facilitated gastrointestinal transit in a dose-dependent manner in conscious mice (Figure 1b), although without pretreatment with amastatin it failed to produce a significant effect (Figure

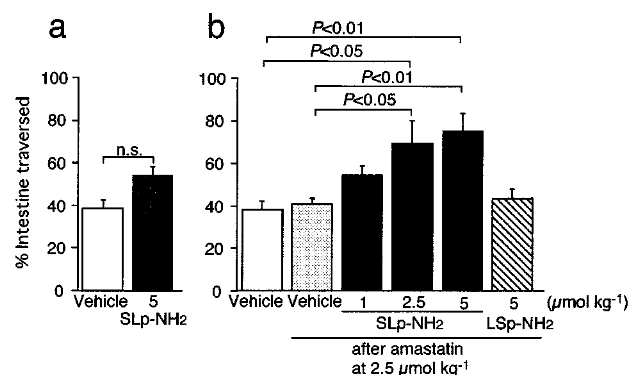

Figure 1 Effects of the PAR-2 agonist on gastrointestinal transit in mice. The PAR-2 agonist SLIGRL-NH_2 (SLp-NH_2) or the control peptide LSIGRL-NH_2 (LSp-NH_2), alone (a) or in combination with i.p. amastatin at 2.5 μmol kg^{-1} (b), were administered i.p. to the mouse. Data represent the mean$\pm$s.e.mean from 4–6 mice. n.s., not significant. Vehicle for amastatin did not modify the gastrointestinal transit.

1a). On the other hand, the PAR-2-inactive control peptide LSIGRL-NH_2 at 5 μmol kg^{-1}, in combination with amastatin, had no effect on gastrointestinal transit (Figure 1b).

The human PAR-1-derived receptor-activating peptide SFLLR-NH_2 that also has a weak agonistic activity toward PAR-2 (Kawabata *et al.*, 1999c), when administered i.p. at 5 and 10 μmol kg^{-1} in combination with amastatin, produced significant increase in gastrointestinal transit in mice (Figure 2a), although without pretreatment with amastatin it had no significant effect (data not shown). On the other hand, the PAR-1-inactive control peptide FSLLR-NH_2 at 10 μmol kg^{-1} had no significant effect (Figure 2a). The PAR-1 agonist analogue TFLLR-NH_2, known to be highly specific for PAR-1 with no PAR-2 activity, by i.p. administration in combination with amastatin, increased gastrointestinal transit in a dose-dependent manner (Figure 2b), although this peptide also required pretreatment with amastatin to produce the effect (data not shown).

Potentiation by apamin of the effects of agonists for PAR-2 and PAR-1 at subeffective doses on gastrointestinal transit in mice

We tested if apamin, an inhibitor of Ca^{2+}-activated, small-conductance K^+ channels, could modify gastrointestinal transit in mice *in vivo*, since activation of PAR-2 and/or PAR-1 can produce relaxation of some of gastrointestinal smooth muscle preparations from mice, rats or guinea-pigs *in vitro* (Cocks *et al.*, 1999; Kawabata *et al.*, 1999b; 2000b). Apamin, when administered i.p. at 0.01 μmol kg^{-1}, did not significantly alter gastrointestinal transit in conscious mice *in vivo* (Figure 3). Preadministration of apamin at the same dose significantly potentiated the effects of the PAR-2 agonist SLIGRL-NH_2 and PAR-1-agonist TFLLR-NH_2 at 1 and 2.5 μmol kg^{-1}, respectively, that had no significant effect on gastrointestinal transit by themselves (Figure 3). On the other hand, the same dose of apamin failed to significantly potentiate the effect of carbachol at 0.055 μmol kg^{-1} that had been confirmed to be a subeffective dose in our preliminary experiments: per cent of intestine traversed was 40.4±1.9, 44.8±2.8 and 48.6±2.0 (n=4) in groups treated with vehicle plus vehicle, vehicle plus carbachol and apamin plus carbachol, respectively.

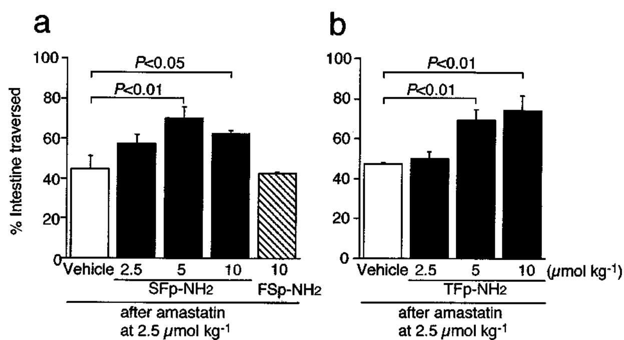

Figure 2 Effects of the PAR-1 agonists on gastrointestinal transit in mice. The human PAR-1-derived peptide SFLLR-NH_2 (SFp-NH_2) or the control peptide FSLLR-NH_2 (FSp-NH_2) (a) and the specific PAR-1 agonist TFLLR-NH_2 (TFp-NH_2) (b), in combination with i.p. amastatin at 2.5 μmol kg^{-1}, were administered i.p. to the mouse. Data represent the mean±s.e.mean from 8–10 (a) or 4–6 (b) mice.

Inhibition by verapamil, an inhibitor of voltage-dependent, L-type Ca^{2+} channels, of the increased gastrointestinal transit produced by agonists for PAR-2 and PAR-1 in mice

We next evaluated the effects of the L-type Ca^{2+} channel inhibitor verapamil on the PAR-2- or PAR-1-mediated increase in gastrointestinal transit in mice *in vivo*, because some *in vitro* studies suggested the involvement of L-type Ca^{2+} channels in contractile responses of gastrointestinal smooth muscle to activation of PAR-2 or PAR-1 (Saifeddine *et al.*, 1996; Zheng *et al.*, 1998; Kawabata *et al.*, 1999b; 2000b). Verapamil, administered s.c. at 61.6 μmol kg^{-1} alone did not affect gastrointestinal transit in mice (Figure 4). The same dose of verapamil completely abolished the facilitating effects of the PAR-2 agonist SLIGRL-NH_2 at 5 μmol kg^{-1} and the PAR-1 agonist TFLLR-NH_2 at 10 μmol kg^{-1} on gastrointestinal transit (Figure 4). On the other hand, verapamil at the same dose significantly but only partially reduced the effect of carbachol at 0.55 μmol kg^{-1}, a dose that

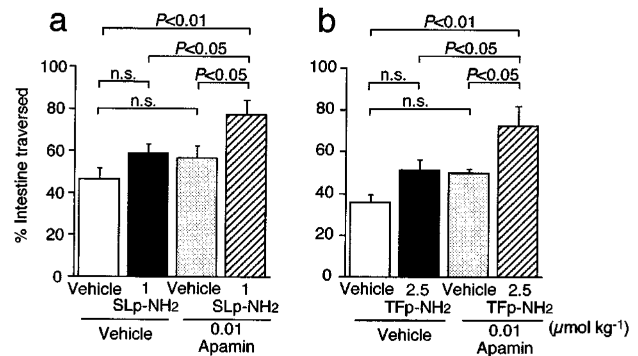

Figure 3 Promoting by apamin of the effects of the PAR-2 agonist SLIGRL-NH_2 (SLp-NH_2) (a) and the PAR-1 agonist TFLLR-NH_2 (TFp-NH_2) (b) at subeffective doses on gastrointestinal transit in mice. Apamin at 0.01 μmol kg^{-1} was administered i.p. to the mouse 6 min before i.p. injection of SLIGRL-NH_2 at 1 μmol kg^{-1} or TFLLR-NH_2 at 2.5 μmol kg^{-1} (5 min before amastatin at 2.5 μmol kg^{-1}). Data represent the mean±s.e.mean from 7–8 (a) or 5–6 (b) mice. n.s., not significant.

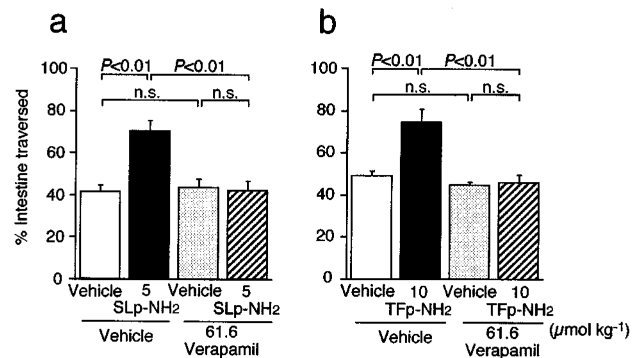

Figure 4 Inhibition by verapamil of the effects of the PAR-2 agonist SLIGRL-NH_2 (SLp-NH_2) (a) and the PAR-1 agonist TFLLR-NH_2 (TFp-NH_2) (b) on gastrointestinal transit in mice. Verapamil at 61.6 μmol kg^{-1} was administered s.c. to the mouse 16 min before i.p. injection of SLIGRL-NH_2 at 5 μmol kg^{-1} or TFLLR-NH_2 at 10 μmol kg^{-1} (15 min before amastatin at 2.5 μmol kg^{-1}). Data represent the mean±s.e.mean from eight mice. n.s., not significant.

had been confirmed to be submaximal in our preliminary experiments, per cent of inhibition being 43.9: per cent of intestine traversed was 36.6 ± 2.8, 88.5 ± 3.1 and 65.7 ± 5.1 ($n = 4 - 6$) in groups treated with vehicle plus vehicle, vehicle plus carbachol and verapamil plus carbachol, respectively.

Lack of effects of genistein, an inhibitor of tyrosine kinase, on the PAR-2- and PAR-1-mediated increase in gastrointestinal transit in mice

In vitro evidence suggests that activation of tyrosine kinase might participate, at least in part, in contraction of gastrointestinal smooth muscle in response to activation of PAR-2 or PAR-1 (Saifeddine *et al*., 1996; Zheng *et al*., 1998; Kawabata *et al*., 1999b; 2000b). We thus finally evaluated if the tyrosine kinase inhibitor genistein could modify the effects of agonists for PAR-2 and PAR-1 on gastrointestinal transit in mice *in vivo*. Neither baseline values nor PAR-2- or PAR-1-mediated enhancement of gastrointestinal transit were significantly altered by s.c. preadministration of genistein at 18.5 μmol kg^{-1} (data not shown).

Discussion

The present study demonstrates that activation of either PAR-2 or PAR-1 increases gastrointestinal transit in mice *in vivo*. Our data also indicate that the augmented gastrointestinal transit *via* PAR-2 and PAR-1 is potentially suppressed by concomitant activation of apamin-sensitive, Ca^{2+}-activated, small-conductance K^+ channels. Furthermore, our results reveal that the effects of the agonists for PAR-2 and PAR-1 are mediated by activation of L-type Ca^{2+} channels, but are independent of tyrosine kinase.

Modulation by PAR-2 and PAR-1 of the smooth muscle motility in the gastrointestinal tract including the oesophagus is very complex, since both excitatory and inhibitory actions of either PAR-2 or PAR-1 agonists on isolated smooth muscle tension have been described (Hollenberg *et al*., 1993; 1997; Saifeddine *et al*., 1996; Corvera *et al*., 1997; Zheng *et al*., 1998; Cocks *et al*., 1999; Kawabata *et al*., 1999b; 2000a, b). The present finding that exogenously applied agonists for PAR-2 or PAR-1 increased gastrointestinal transit suggests roles of these receptors in stimulating gastrointestinal motility. As PAR-2 and PAR-1 might be activated by agonist enzymes, i.e. trypsin, tryptase or coagulation factors VIIa and Xa for PAR-2 and thrombin for PAR-1 (Dery *et al*., 1998; Hollenberg, 1999; Kawabata & Kuroda, 2000; Camerer *et al*., 2000), in the early stage of inflammation or tissue-injury, the increased gastrointestinal transit due to activation of PAR-2 or PAR-1 might occur only in pathological conditions. A similar role of PAR-2 has been suggested in the pancreas where PAR-2 mediates pancreatic juice secretion *in vivo* as well as ductal secretion *in vitro* (Nguyen *et al*., 1999; Kawabata *et al*., 2000d). Taken together, these observations suggest that PARs might work as 'sentries' for inflammation, as proposed elsewhere (Cocks & Moffatt, 2000). However, our hypothesis remains to be demonstrated by more detailed studies using more potent, selective non-peptide agonists and antagonists for PAR-1 and PAR-2 in future.

PAR-1, but not PAR-2, activates apamin-sensitive K^+ channels, resulting in relaxation of isolated rat duodenal smooth muscle (Kawabata *et al*., 1999b; 2000b), and both PAR-2 and PAR-1 mediate apamin-sensitive relaxation of mouse gastric fundus precontracted with carbachol *in vitro* (Cocks *et al*., 1999). These reports predict that agonists for PAR-2 or PAR-1, given *in vivo*, would suppress gastrointestinal transit. The present study, however, excludes this possibility and implies that apamin-sensitive K^+ channels are potentially activated depending upon activation of PAR-2 or PAR-1 *in vivo*, which is overcome by their excitatory effects through distinct mechanisms. The physiological significance of the potential inhibitory properties of PAR-2 and PAR-1 through apamin-sensitive K^+ channels in modulation of gastrointestinal transit remains to be investigated.

That the increased gastrointestinal transit mediated by PAR-2 or PAR-1 was completely abolished by verapamil is consistent with the previous *in vitro* evidence that the contractile responses of gastrointestinal smooth muscle to agonists for PAR-2 or PAR-1 are largely dependent on activation of L-type Ca^{2+} channels (Saifeddine *et al*., 1996; Zheng *et al*., 1998; Kawabata *et al*., 1999b; 2000b). The finding that verapamil did not suppress the basal gastrointestinal transit (see Figure 4) is in agreement with the work by di Carlo *et al*. (1993), while some other studies have revealed decreased gastrointestinal transit following verapamil (Shah *et al*., 1987; Calignano *et al*., 1992). The effectiveness of verapamil in the resting state might vary with experimental conditions such as the strain or size of mice employed. There is *in vitro* evidence that tyrosine kinase plays a role in the contraction of gastrointestinal longitudinal smooth muscle in response to activation of PAR-1 or PAR-2 (Saifeddine *et al*., 1996; Zheng *et al*., 1998; Kawabata *et al*., 1999b; 2000b). However, our data suggest that tyrosine kinase does not contribute to the modulation by PAR-1 and PAR-2 of gastrointestinal transit *in vivo*.

Our present data that PAR-2 and PAR-1 modulated gastrointestinal transit *in vivo* further support the importance of the roles of these receptors in the gastrointestinal systems, especially under pathophysiological conditions such as inflammation.

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