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Modulation by protease-activated receptors of the rat duodenal motility *in vitro*: possible mechanisms underlying the evoked contraction and relaxation

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1 The present study examined effects of agonist enzymes and receptor-activating peptides for protease-activated receptors (PARs) on duodenal motility in the rat, and also investigated possible mechanisms underlying the evoked responses.

2 Thrombin at 0.03–0.1 μM and the PAR-1-activating peptide SFLLR-NH_2 at 3–100 μM or TFLLR-NH_2 at 10–50 μM produced a dual action, relaxation followed by contraction of the duodenal longitudinal muscle. The PAR-2-activating peptide SLIGRL-NH_2 at 10–100 μM elicited only small contraction. Trypsin at 0.08 μM induced small contraction, or relaxation followed by contraction, depending on preparations. The PAR-4-activating peptide GYPGKF-NH_2 at 1000 μM exhibited no effect.

3 The contractile responses of the duodenal strips to TFLLR-NH_2 and to SLIGRL-NH_2 were partially attenuated by the L-type calcium channel blocker nifedipine (1 μM), the protein kinase C inhibitor GF109203X (1 μM) and the tyrosine kinase inhibitor genistein (15 μM), but were resistant to indomethacin (3 μM) and tetrodotoxin (1–10 μM).

4 The relaxation of the preparations exerted by TFLLR-NH_2 was unaffected by indomethacin (3 μM), propranolol (5 μM), N^G-nitro-L-arginine methyl ester (100 μM) and tetrodotoxin (1–10 μM). This relaxation was resistant to either GF109203X (1 μM) or genistein (15 μM), but was, remarkably, attenuated by combined application of these two kinase inhibitors.

5 Apamin (0.1 μM), an inhibitor of calcium-activated, small-conductance potassium channels, but not charybdotoxin (0.1 μM), completely abolished the PAR-1-mediated duodenal relaxation, and significantly enhanced the PAR-1-mediated contraction.

6 These findings demonstrate that PAR-1 plays a dual role, suppression and facilitation of smooth muscle motility in the rat duodenum, while PAR-2 plays a minor excitatory role in the muscle, and that PAR-4 is not involved in the duodenal tension modulation. The results also suggest that the contractile responses to PAR-1 and PAR-2 activation are mediated, in part, by activation of L-type calcium channels, protein kinase C and tyrosine kinase, and that the relaxation response to PAR-1 activation occurs *via* activation of apamin-sensitive, but charybdotoxin-insensitive, potassium channels, in which both protein kinase C and tyrosine kinase might be involved synergistically.

Keywords: Protease (proteinase)-activated receptor (PAR); thrombin; trypsin; rat duodenum; motility; contraction; relaxation; protein kinase C; tyrosine kinase; potassium channel

Abbreviations: L-NAME, N^G-nitro-L-arginine methyl ester; PAR, protease-activated receptor

Introduction

The protease-activated receptor (PAR), a unique family of G-protein coupled, seven trans-membrane domain receptors, is activated by the exposed 'tethered ligand' following proteolytic unmasking of the extracellular N-terminal cryptic receptor-activating sequences (for review, see Dery *et al*., 1998). Among four members of this family that have been cloned, PAR-1, PAR-3 and PAR-4 are activated by thrombin, while PAR-2 is activated by trypsin or tryptase (Vu *et al*., 1991; Nystedt *et al*., 1994; Molino *et al*., 1997; Ishihara *et al*., 1997; Kahn *et al*., 1998; Xu *et al*., 1998). Synthetic peptides consisting of five or six amino acids based on the N-terminal sequences of the tethered ligands of PAR-1, PAR-2 and PAR-4, when applied exogenously, are capable of fully activating the parent receptors (Vu *et al*., 1991; Nystedt *et al*., 1994; Dery *et al*., 1998; Kahn *et al*., 1998; Xu *et al*., 1998), whereas PAR-3 does not respond to the presumed receptor-activating peptides (Ishihara *et al*., 1997).

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PARs appear to be involved in a number of physiological or pathophysiological events including platelet aggregation (Vu *et al*., 1991; Ishihara *et al*., 1997; Kahn *et al*., 1998; Cicala *et al*., 1999), inflammation (Cirino *et al*., 1996; Kawabata *et al*., 1998; 1999a; Vergnolle *et al*., 1999), vascular contraction/relaxation (Muramatsu *et al*., 1992; Laniyonu & Hollenberg, 1995; Saifeddine *et al*., 1996; Moffatt & Cocks, 1998; Sobey & Cocks, 1998), neurodegeneration/neuroprotection (Smith-Swintosky *et al*., 1997; Donovan & Cunningham, 1998), gastrointestinal functions (Hollenberg *et al*., 1992; Saifeddine *et al*., 1996; Corvera *et al*., 1997; Kong *et al*., 1997; Zheng *et al*., 1998; Nguyen *et al*., 1999), etc. Agonists for PAR-1 and for PAR-2 elicit contraction of the gastric longitudinal smooth muscle isolated from rats or guinea-pigs, an effect being abolished by indomethacin (Hollenberg *et al*., 1992; Saifeddine *et al*., 1996; Zheng *et al*., 1998). PAR-2 mediates activation of pancreatic duct epithelial cell ion channels in the rat (Nguyen *et al*., 1999). PAR-2 activation inhibits spontaneous rhythmic contractions of the rat colonic strips in an indomethacin-

resistant manner (Corvera *et al*., 1997). Trypsin and PAR-2-activating peptides increase secretion of eicosanoids (Kong *et al*., 1997) and also stimulate ionic transport *via* a prostanoid-mediated mechanisms (Vergnolle *et al*., 1998) in the rat jejunum. Thus, PAR-1 and PAR-2 may play a key role in digestive systems.

In the present study, to clarify the role of PARs in modulation of motility of the small intestine, we evaluated effects of activation of PARs on the tension oscillation of the rat duodenum, using thrombin, trypsin and receptor-activating peptides targeted toward PAR-1, PAR-2 and PAR-4 (Kawabata *et al*., 1997; 1999b; Hollenberg *et al*., 1997; Kahn *et al*., 1998).

Methods

Tissue preparation and isometric recording

Male Wistar rats weighing 250–350 g (Japan SLC. Inc., Japan) were decapitated under pentobarbital (50 mg kg^{-1}, i.p.) anaesthesia, and after exsanguination, 1-cm long segments of the duodenum isolated from the rat were prepared. The cylindrical segment the upper and lower ends of which were set with micro-pinch holders at a side opposite to the mesentery, was suspended in an organ bath containing 4 ml of gassed (95% O_2/5% CO_2) Krebs-Henseleit solution pH 7.4 of the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; KH_2PO_4, 1.2 and glucose, 10. The solution was maintained at 37°C, and the tissue was allowed to equilibrate for 1 h under a resting tension of 10 mN. Mechanical activity of the longitudinal muscle of the duodenal segments was recorded isometrically through a force-displacement transducer (UL-10GR, Minebea Co., Ltd., Japan). As the duodenal tissue exhibited spontaneous motility, the agonists-evoked contraction and relaxation were defined as changes in the resting tone (the bottom level of the tension oscillation). The contractile responses are expressed as a percentage (% KCl) of the contraction caused by 50 mM KCl in the same preparation, and the relaxation responses are presented as a percentage (% papaverine) of the relaxation caused by 100 μM papaverine. In some experiments, the minimal amplitude of the spontaneous motility following drug addition was measured and expressed as a percentage (% control) of the mean amplitude of spontaneous contraction for 5 min immediately before drug addition.

Experimental protocol

After the equilibration period, the preparations were exposed, a few times at 30-min intervals, to a 50 mM K^+-containing solution that was made by replacing NaCl in the Krebs-Henseleit solution with required KCl. Then, receptor-activating peptides for PARs at one of various concentrations (0.1–200 μM) were routinely applied to the preparations at 45-min intervals with tissue washes about every 10–15 min. Agonist peptides employed were as follows: SFLLR-NH_2, a human PAR-1-derived PAR-1 agonist that has some activity on PAR-2, TFLLR-NH_2, a highly specific PAR-1 agonist, SLIGRL-NH_2, a murine PAR-2-derived specific PAR-2 agonist, GYPGKF-NH_2, a murine PAR-4-derived PAR-4 agonist (Kawabata *et al*., 1997; 1999b; Hollenberg *et al*., 1997; Kahn *et al*., 1998). In some experiments, to confirm the specificity of the effect, the inactive control peptides, FSLLR-NH_2, LSIGRL-NH_2 and GAPGKF-NH_2, for PAR-1, PAR-2 and PAR-4, respectively, were applied to the preparations, being followed by addition of each agonist. The assay of peptide activity was always performed in the presence of 10 μM amastatin, an inhibitor of aminopeptidase that degrades peptides, which was added to the organ bath 1 min before peptide application. In the preliminary experiments, amastatin itself did not affect the duodenal tension. Thrombin and trypsin were applied only once to each tissue preparation because after a single application of each protease, the response did not completely recover, even by repeated washes, within 2 h. In experiments to determine mechanisms underlying duodenal responses mediated by PARs, activity of PARs-activating peptides was assessed in the presence and absence of the following inhibitors or antagonists at appropriate concentrations as suggested previously (Laniyonu & Hollenberg, 1995; Saifeddine *et al*., 1996; Corvera *et al*., 1997; Edwards *et al*., 1998): the L-type voltage-dependent calcium channel blocker nifedipine at 1 μM, the cyclo-oxygenase inhibitor indomethacin at 3 μM, the protein kinase C inhibitor GF109203X at 1 μM, the tyrosine kinase inhibitor genistein at 15 μM, the β-adrenoceptor antagonist propranolol at 5 μM, the nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) at 100 μM, the sodium channel blocker tetrodotoxin at 1 and 10 μM, the small-conductance, calcium-activated potassium channel blocker apamin at 0.1 μM, and the large- or intermediate-conductance, calcium-activated potassium channel blocker charybdotoxin at 0.1 μM. These drugs were added to the organ bath, 15 min prior to the addition of agonists for PARs. After completion of the experiments, the duodenal strips were exposed to 100 μM papaverine to obtain the maximal relaxation.

Reagents employed

PARs-related peptides were prepared by a standard solid phase synthesis procedure either by Dr D. McMaster and his colleagues of the Peptide Synthesis Core Facility at the University of Calgary (Canada) or by ourselves. The concentration, purity and composition of the peptides were determined by high-performance liquid chromatography, mass spectrometry and quantitative amino acid analysis. Human thrombin, porcine trypsin, nifedipine, L-NAME hydrochloride, apamin and tetrodotoxin were purchased from Sigma (U.S.A.), and GF109203X and genistein were from Research Biochemicals International (U.S.A.). Propranolol was obtained from Tokyo Kasei (Japan), charybdotoxin was provided from Peptide Institute Inc. (Japan), and indomethacin was from Wako Pure Chemicals (Japan). GF109203X and genistein were dissolved in 50% DMSO and diluted with distilled water, and indomethacin was dissolved in 5 mM Na_2CO_3 immediately before use. All other chemicals were dissolved in distilled water. In the control experiments, each vehicle was added to the organ bath.

Statistical analysis

All data obtained are expressed as means ± s.e.mean. The differences in the agonists-induced responses in the absence (control) and presence of inhibitors or antagonists were analysed by the paired *t*-test, and considered significant when $P<0.05$.

Results

Effects of thrombin, trypsin and receptor-activating peptides for PARs on the tension oscillation of the rat duodenal strips

The isolated duodenal strips exhibited spontaneous contractions, the magnitude of which varied with preparations. Thrombin, an enzyme activating PAR-1, PAR-3 and PAR-4, at 0.2 μM, elicited relaxation and transient suppression of spontaneous motility, followed by contraction in the rat duodenal segments. The relaxation occurred within 20 s after addition, disappearing in about 1 min, and then the small contraction slowly developed, disappearing in about 5 min (Figure 1a). The human PAR-1-derived PAR-1 agonist SFLLR-NH_2 at 100 μM also produced a similar, but relatively rapid, dual action, the magnitude of both relaxation and contraction being much greater than that by thrombin, although the inactive control peptide FSLLR-NH_2 at the same concentration had no effect (Figure 1b); the results were the same even if the preparations were washed between the two additions of peptides (data not shown). The highly selective PAR-1 agonist TFLLR-NH_2 at 10 and 100 μM also mimicked the dual effect of SFLLR-NH_2. This peptide at low concentrations, 1–3 μM, suppressed the duodenal motility without subsequent contraction, an effect disappearing within 2 min (Figure 1c). Trypsin, an enzyme activating PAR-2, at 0.08 μM elicited two distinct types of responses depending on preparations: one is slowly developing small contraction (four out of eight preparations), and the other is relaxation followed by contraction (four out of eight) (Figure 1d). In the former case, the contraction developed about 1 min after addition, lasting for 5–10 min, and in the latter case, the response was similar to that due to thrombin (Figure 1a). The PAR-2-selective agonist SLIGRL-NH_2 at 100 μM produced slowly developing small contraction after a latency of 20–50 s, without preceding relaxation in the duodenal segments, while the inactive control peptide LSIGRL-NH_2 at the same concentration failed to contract the preparations (Figure 1e). The PAR-4 agonist GYPGKF-NH_2, like the inactive control peptide GAYPGKF-NH_2, even at 1000 μM, produced neither contraction nor relaxation in the rat duodenum (Figure 1f). The results were the same, even if the preparations were washed between the two additions of peptides in Figure 1e,f (data not shown).

The contractile responses to SFLLR-NH_2 and to TFLLR-NH_2 were concentration-dependent in ranges of 3–100 μM and 10–50 μM, respectively. The concentration-response curve for SFLLR-NH_2 was on the left of that for TFLLR-NH_2 (Figure 2, the top panel); significant ($P<0.05$) difference was detected at 10 μM of the two PAR-1 agonists. The relaxation and suppression of spontaneous contraction exerted by SFLLR-NH_2 and by TFLLR-NH_2 in the duodenum were detected at lower concentrations, 0.1–10 μM, and the concentration-response curves for the two peptides overlapped each other (Figure 2, the lower two panels). The contractile response to SLIGRL-NH_2 was obtained at a range of 3–100 μM, while there was neither relaxation nor significant suppression of spontaneous contraction at these concentrations (Figure 2). The control peptides, FSLLR-NH_2 and LSIGRL-NH_2 were ineffective in the preparations, although the latter peptide slightly (but not significantly at any concentrations) reduced spontaneous contraction in the duodenum (Figure 2). The contractile or relaxing responses to thrombin and to trypsin were detected at much lower concentrations, 0.01–0.2 μM, whereas the magnitude of responses to the enzymes was much smaller than that by receptor-activating peptides for PAR-1 (Figure 2).

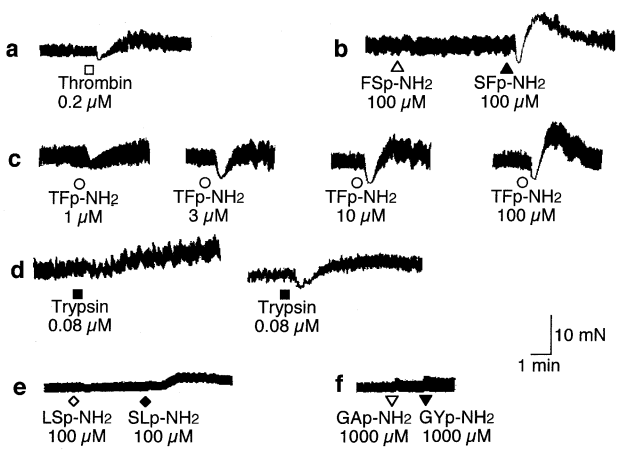

Figure 1 Representative recordings of tension oscillation of the rat duodenal longitudinal smooth muscle in response to thrombin (a), the PAR-1-activating peptide SFLLR-NH_2 (SFp-NH_2) or TFLLR-NH_2 (TFp-NH_2) (b, c), trypsin (d), the PAR-2-activating peptide SLIGRL-NH_2 (SLp-NH_2) (e), the PAR-4-activating peptide GYPGKF-NH_2 (GYp-NH_2) (f), and the respective inactive control peptide FSLLR-NH_2 (FSp-NH_2) (b), LSIGRL-NH_2 (LSp-NH_2) (e) or GAPGKF-NH_2 (GAp-NH_2) (f). In (b), (e) and (f), the second peptide was added without washing after the first peptide application.

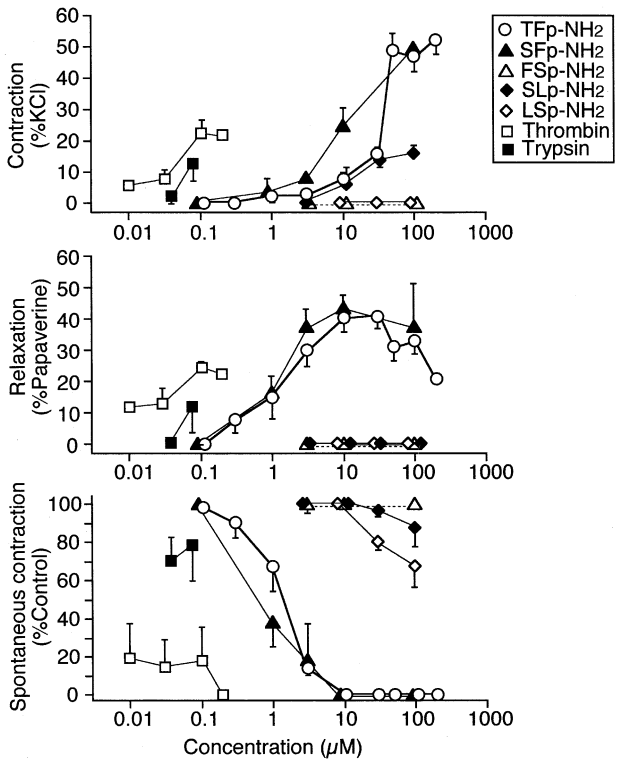

Figure 2 Concentration-effect curves for the contraction, relaxation and suppression of spontaneous contraction exerted by the PAR-1 agonist TFLLR-NH_2 (TFp-NH_2) or SFLLR-NH_2 (SFp-NH_2), the PAR-1-inactive control peptide FSLLR-NH_2 (FSp-NH_2), the PAR-2-agonist SLIGRL-NH_2 (SLp-NH_2), the PAR-2-inactive control peptide LSIGRL-NH_2 (LSp-NH_2), thrombin and trypsin. Data represent the mean ± s.e.mean from four distinct experiments. The maximal contraction (mN) caused by 50 mM KCl was 18.77 ± 0.90 ($n=36$) in the preparations employed.

Effects of blockers or inhibitors of L-type calcium channel, cyclo-oxygenase, protein kinase C and tyrosine kinase on the duodenal responses to receptor-activating peptides for PAR-1 and for PAR-2

The L-type calcium channel blocker nifedipine at 1 μM reduced the PAR-1 agonist TFLLR-NH_2 (50 μM)-induced contraction by 60% and the PAR-2 agonist SLIGRL-NH_2 (100 μM)-induced contraction by 50% (Figure 3). As nifedipine, by itself, almost maximally reduced the resting tone of the duodenal strips, we could not see the effect of nifedipine on the PAR-1-mediated relaxation. The cyclo-oxygenase inhibitor indomethacin at 3 μM, the protein kinase C inhibitor GF109203X at 1 μM and the tyrosine kinase inhibitor genistein at 15 μM did not significantly affect the spontaneous motility by themselves; the post-drug value (% control) relative to the pre-drug motility was 115.4 ± 13.7, 97.6 ± 3.7 and 75.5 ± 5.7 ($n=4$), respectively. The contractile effect of TFLLR-NH_2 (50 μM) was resistant to indomethacin at 3 μM, but was partially blocked by GF109203X at 1 μM and by genistein at 15 μM (Figure 4a–c, left). The suppressive effects of the two kinase inhibitors, when co-administered, on the evoked contraction were not additive, but even less than the effect of each inhibitor (Figure 4d, left). In contrast, the relaxation exerted by TFLLR-NH_2 was not altered by Indomethacin, GF109203X or genistein (Figure 4a–c, right). Surprisingly, however this relaxation was significantly reduced by combined administration of GF109203X and genistein (Figure 4d, right). The PAR-2 agonist SLIGRL-NH_2 (100 μM)-induced small contraction showed a property similar to that by PAR-1 activation. GF109203X or genistein, but not Indomethacin, partially suppressed the PAR-mediated contraction, whereas, remarkably, the effects of the two kinase inhibitors, when co-administered, were rather reduced (Figure 5).

Effects of propranolol, L-NAME and tetrodotoxin on the relaxation of the duodenal strips induced by the PAR-1-activating peptide

To clarify the mechanisms underlying the relaxation produced by the PAR-1-activating peptide, we evaluated effects of propranolol, L-NAME and tetrodotoxin on the effect of TFLLR-NH_2 at 50 μM. Propranolol at 5 μM that did not significantly modify the spontaneous motility by itself (the post-drug value (% control) was 79.6 ± 0.8, $n=4$), had no effect on the TFLLR-NH_2 (50 μM)-induced relaxation of the duodenum (Figure 6a). L-NAME at 100 μM and tetrodotoxin at 10 μM, by themselves, significantly ($P<0.01$ and $P<0.05$, respectively) increased spontaneous contractions in the duodenal preparations (the post-drug value (% control) was 129.2 ± 5.8 and 146.7 ± 7.3, respectively, $n=4$), in agreement with the previous findings (Martinez-Cuesta *et al.*, 1996; Corvera *et al.*, 1997). L-NAME at 100 μM as well as tetrodotoxin at 10 μM (and also at 1 μM (data not shown)) did not reduce the PAR-1-mediated relaxation (Figure 6b,c). It is also of note that tetrodotoxin did not abolish the contraction evoked by the PAR-1 agonist TFLLR-NH_2 (Figure 6c) and by the PAR-2 agonist SLIGRL-NH_2 (data

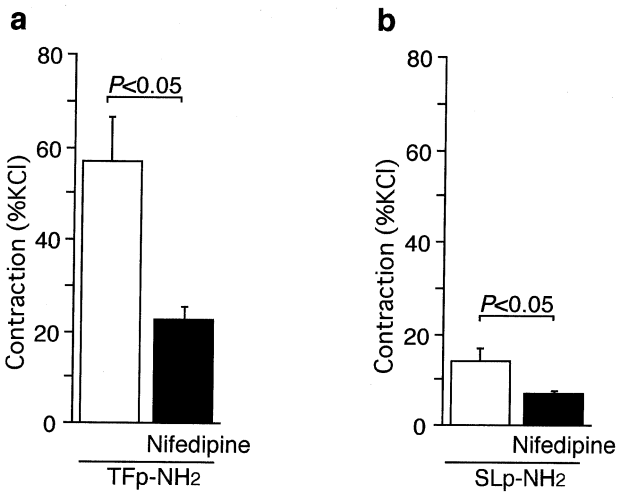

Figure 3 Effect of nifedipine on the contractile effects of agonist peptides for PAR-1 (a) and PAR-2 (b) in the rat duodenal segments. After monitoring a control response to TFLLR-NH_2 (TFp-NH_2) at 50 μM (a) or to SLIGRL-NH_2 (SLp-NH_2) at 100 μM (b), the preparations were washed and, thereafter, rechallenged with the same agonists 15 min following addition of nifedipine at 1 μM. Data represent the mean $\pm$ s.e.mean from four distinct experiments.

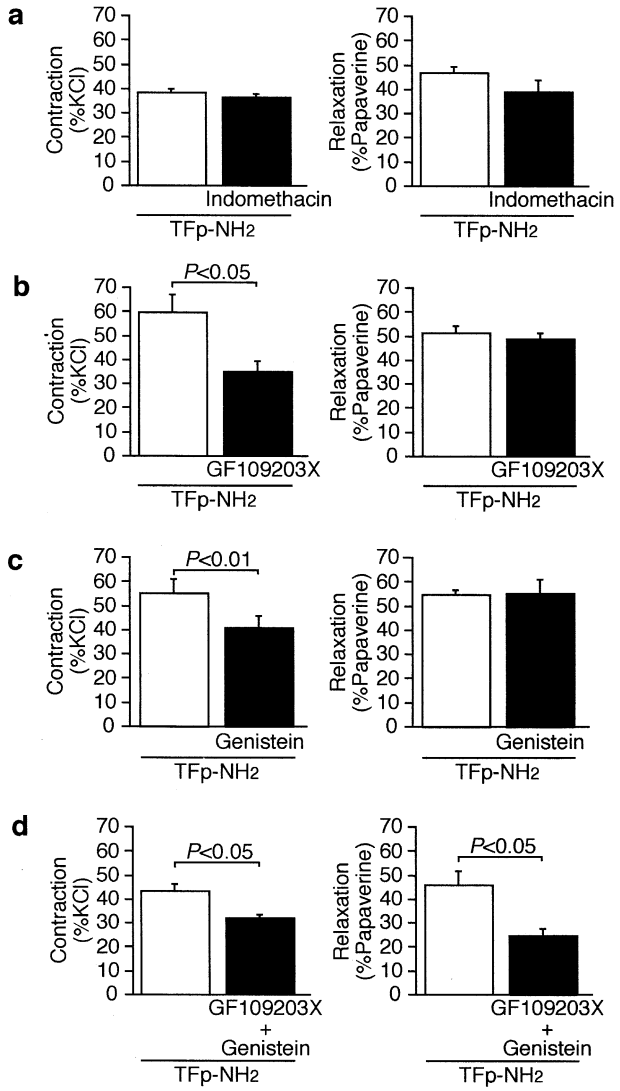

Figure 4 Effects of indomethacin (a), GF109203X (b), genistein (c) and GF109293X plus genistein (d) on the contractile (left panels) and relaxation (right panels) responses of the rat duodenal muscle to the PAR-1-activating peptide. After monitoring a control response to TFLLR-NH_2 (TFp-NH_2) at 50 μM, the preparations were washed and, thereafter, rechallenged with the same agonist 15 min following addition of indomethacin at 3 μM (a), GF109203X 1 μM (b), genistein at 15 μM (c) or GF109203X plus genistein (d). Data represent the mean $\pm$ s.e.mean from four distinct experiments.

not shown) in the duodenal preparations, being comparable with the findings in gastric or aortic smooth muscle preparations (Laniyonu *et al*., 1995; Saifeddine *et al*., 1996).

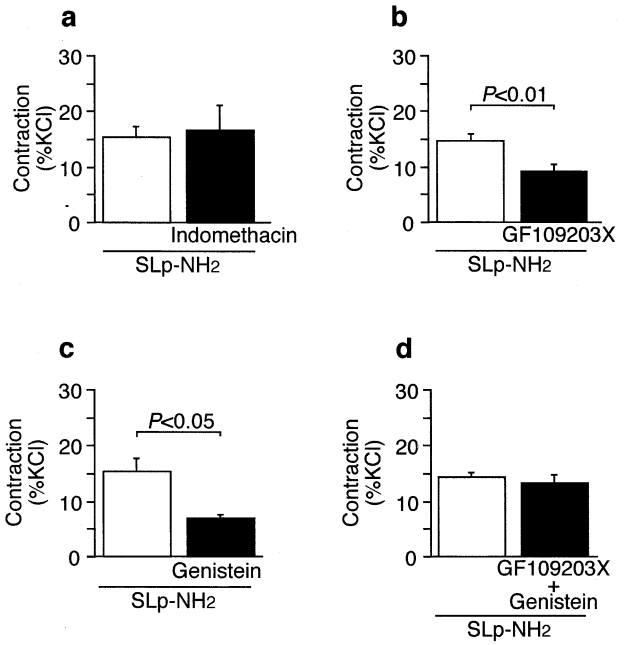

Figure 5 Effects on indomethacin (a), GF109203X (b), genistein (c) and GF109203X plus genistein (d) on the contraction of the rat duodenal strips triggered by the PAR-2-activating peptide SLIGRL-NH_2. After monitoring a control response to SLIGRL-NH_2 (SLp-NH_2) at 100 μM, the preparations were washed and, thereafter rechallenged with the same agonist 15 min following addition of indomethacin at 3 μM (a), GF109203X at 1 μM (b), genistein at 15 μM (c) or GF109203X plus genistein (d). Data represent the mean$\pm$s.e.mean from four (for experiments using indomethacin and genistein) or eight (for experiments using GF109203X, and GF109203X plus genistein) distinct experiments.

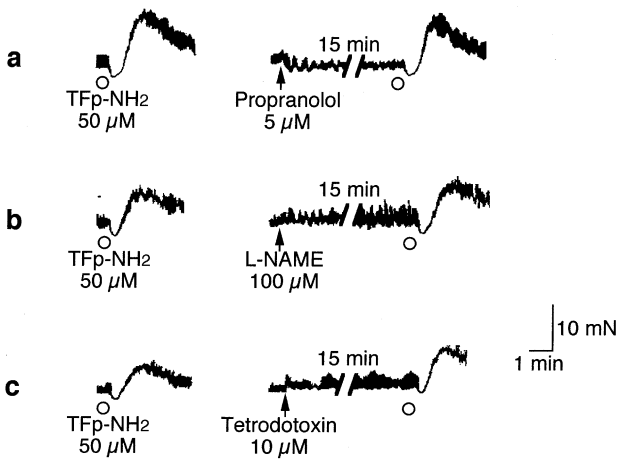

Figure 6 Effects of propranolol (a), L-NAME (b) and tetrodotoxin (c) on the responses of the rat duodenal preparations to the PAR-1-activating peptide. After monitoring a control response to TFLLR-NH_2 (TFp-NH_2) at 50 μM (recordings on the left side), the preparations were washed and, thereafter, rechallenged with the same agonist 15 min following addition of propranolol at 5 μM (a), L-NAME at 100 μM (b) and tetrodotoxin at 10 μM (c) (recordings on the right side). The recordings are representative of similar results obtained from four distinct experiments.

Effects of inhibitors of potassium channels on the PAR-1-mediated relaxation of the rat duodenal preparations

Finally, we examined involvement of potassium channels in the PAR-1-mediated relaxation of rat duodenal muscle. Apamin, an inhibitor of the calcium-activated, small-conductance potassium channel, at 0.1 μM, by itself, strongly and significantly ($P<0.05$) augmented the spontaneous contractions in the rat duodenal strips (Figure 7a); the post-drug value (% control) was 220.1$\pm$11.4 ($n=4$). Apamin at this concentration significantly enhanced the contraction due to TFLLR-NH_2 at 50 μM (Figure 7a,b), but did not affect the contraction of carbachol at an equipotential concentration, 0.3 μM (Figure 7b). Remarkably, apamin almost completely abolished TFLLR-NH_2-induced relaxation in the rat duodenal muscle strips (Figure 7a,b), whereas it did not modify the

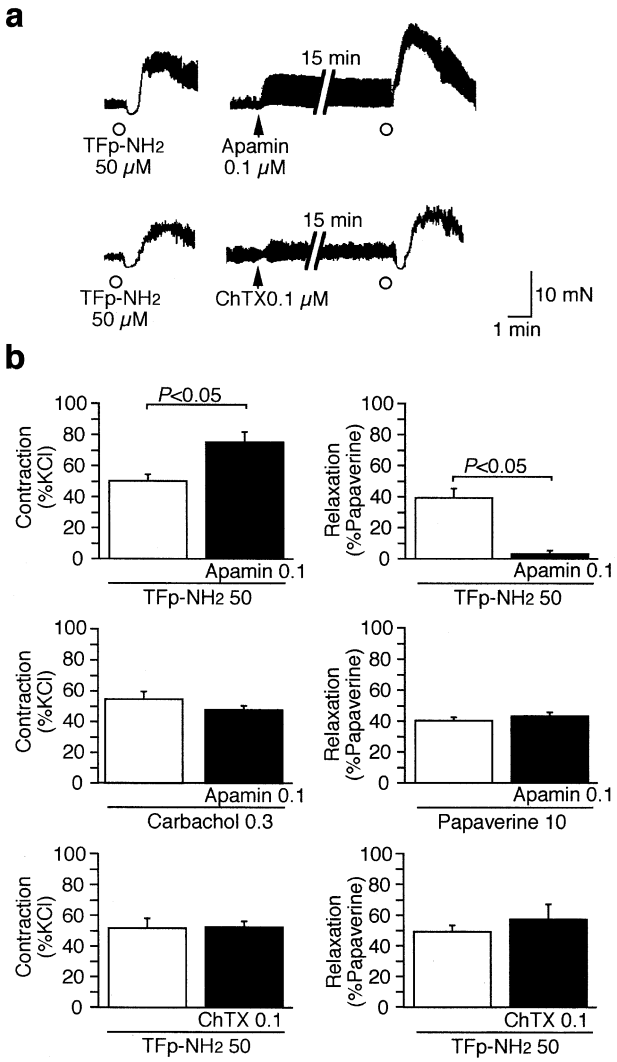

Figure 7 Effects of potassium channel blockers on the responses of the rat duodenal smooth muscle to the PAR-1-activating peptide. After monitoring a control response to TFLLR-NH_2 (TFp-NH_2) at 50 μM (recordings on the left side in (a)), the preparations were washed and, thereafter, rechallenged with the same agonist 15 min following addition of apamin at 0.1 μM or charybdotoxin (ChTX) at 0.1 μM (recordings on the right side in (a). Effects of apamin on responses to carbachol at 0.3 μM and to papaverine at 10 μM were also examined in the same manner. The recordings in (a) are representative of four similar recordings in distinct preparations. Data in (b) represent the mean$\pm$s.e.mean from four distinct experiments.

relaxation due to papaverine at an equipotential concentration, 10 μM (Figure 7b). In contrast, charybdotoxin, an inhibitor of the calcium-activated, intermediate- and large-conductance potassium channels, at 0.1 μM, did not significantly alter the contraction and relaxation produced by TFLLR-NH_2 in the duodenal strips (Figure 7a,b), although this toxin, by itself, also tended to augment the spontaneous contractions in the preparations (Figure 7a); the post-drug value (% control) was 127.3 ± 5.7 ($n=4$), an effect being non-significant.

Discussion

The present study demonstrates that PAR-1 activation evokes relaxation followed by contraction of the rat duodenal longitudinal smooth muscle, while PAR-2 activation elicits only small contraction of the muscle, and also reveals that PAR-4 activation does not alter duodenal motility. The findings that the contractile responses to agonists for PAR-1 and for PAR-2 were reduced, to a certain extent, by nifedipine, GF109203X and genistein, but not by indomethacin and tetrodotoxin, suggest involvement, at least in part, of L-type calcium channels, protein kinase C and tyrosine kinase, but not of cyclo-oxygenase and enteric nerves, in both PAR-1 and PAR-2-mediated duodenal contraction. Our results also suggest that the PAR-1-mediated relaxation of the duodenal muscle is attributable to activation of the calcium-activated, small-conductance potassium channels that are sensitive to apamin but insensitive to charybdotoxin, while it is independent of cyclo-oxygenase and neuronal elements including nitric oxide and β-adrenoceptor systems. Furthermore, that the evoked relaxation due to PAR-1 activation was suppressed by GF109203X plus genistein, but not by each of them, may imply that both protein kinase C and tyrosine kinase might be involved synergistically in the activation of the apamin-sensitive potassium channels.

The rat duodenal longitudinal muscle exhibited essentially distinct responses to activation of PAR-1 and to activation of PAR-2 in the present study. The finding that the maximal contractile and relaxation responses to thrombin in the rat duodenal strips were smaller than those to PAR-1-activating peptides is consistent with the previous evidence concerning contractile responses of the guinea-pig gastric longitudinal smooth muscle (Hollenberg *et al*., 1992) and of the rat aorta (Laniyonu *et al*., 1995), although the reason why thrombin was not fully active is not clear at present. The concentration-contractile response curve for SFLLR-NH_2 was on the left of that for TFLLR-NH_2, whereas the concentration-effect curves for the peptides-induced relaxation and suppression of spontaneous motility overlapped each other. This discrepancy may be explained by activation of PAR-2 due to SFLLR-NH_2, since the PAR-1/PAR-2 selectivity of this peptide is only four as assessed by a receptor desensitization assay utilizing a human embryonic kidney cell calcium signalling (Kawabata *et al*., 1999). It was a little confusing that the duodenal responses to trypsin varied with preparations. Some duodenal strips (four out of eight) that revealed a dual action in response to trypsin at 0.08 μM as seen following PAR-1 activation, might express a greater amount of PAR-1 than the other strips that revealed only contraction as seen following the PAR-2 agonist SLIGRL-NH_2, because trypsin, an agonist enzyme for PAR-2, at this concentration, is also capable of weakly activating PAR-1 (Kawabata *et al*., 1999).

The smooth muscle responses to activation of PAR-1 and PAR-2 are greatly distinct depending on the tissues. PAR-1 activation directly contracts rat vascular smooth muscle, but relaxes it *via* release of nitric oxide from the endothelium (Muramatsu *et al*., 1992; Laniyonu & Hollenberg, 1995; Hollenberg *et al*., 1997). PAR-2 activation induces only endothelial nitric oxide-dependent relaxation of rat vascular tissues (Saifeddine *et al*., 1996; Hollenberg *et al*., 1997; Sobey & Cocks, 1998), although there is exceptional evidence that PAR-2 activation directly contracts mouse renal arteries (Moffatt & Cocks, 1998). The PAR-1-mediated contractile response in rat aorta is partially dependent on activation of L-type calcium channels and protein kinase C, but is independent of activation of cyclo-oxygenase or tyrosine kinase (Laniyonu & Hollenberg, 1995). Activation of PAR-1 and of PAR-2 results in contraction of gastric longitudinal smooth muscle isolated from rats or guinea-pigs (Hollenberg *et al*., 1992; Saifeddine *et al*., 1996; Zheng *et al*., 1998). The PAR-1-mediated gastric contraction occurs through activation of L-type calcium channels, cyclo-oxygenase, protein kinase C and tyrosine kinase (Hollenberg *et al*., 1992; Zheng *et al*., 1998), whereas the PAR-2-mediated response is independent of protein kinase C (Saifeddine *et al*., 1996). Thus, the signal transduction pathways activated by PAR-1 and PAR-2 in the smooth muscles appear to be distinct depending on the tissues. Our present data imply that the contraction of rat duodenal longitudinal smooth muscle triggered by activation of PAR-1 and PAR-2 is mediated, at least in part, by activation of L-type calcium channels, protein kinase C and tyrosine kinase, but is independent of cyclo-oxygenase. The lack of inhibition by indomethacin of the PAR-2-mediated duodenal contraction was unexpected, because it had been reported that PAR-2 agonists increase secretion of eicosanoids (Kong *et al*., 1997) and also stimulate ionic transport *via* a prostanoid-mediated mechanisms (Vergnolle *et al*., 1998) in the rat jejunum.

It is especially of interest that PAR-1 activation also induced transient suppression of motility of the rat duodenal strips. Corvera *et al*. (1997) have provided similar findings that PAR-2 activation abolishes spontaneous contraction of rat colonic strips, which is resistant to indomethacin, L-NAME and tetrodotoxin. The PAR-1-mediated relaxation of duodenal preparations in the present study was resistant to indomethacin, propranolol, L-NAME and tetrodotoxin. However, this response was almost completely blocked by apamin, an inhibitor of calcium-activated, small-conductance potassium channels, but was not altered by charybdotoxin, an inhibitor of calcium-activated, intermediate- or large-conductance potassium channels. Apamin, but not charybdotoxin, also facilitated the PAR-1-mediated contraction. The specificity of the effect of apamin on PAR-1-mediated responses is demonstrated by the finding that apamin did not alter the contraction induced by carbachol and the relaxation induced by papaverine. Collectively, PAR-1 activation appears to trigger activation of apamin-sensitive, but charybdotoxin-insensitive, potassium channels, resulting in transient relaxation and suppression of contraction that is mediated partially by activation of L-type calcium channels, protein kinase C and tyrosine kinase. Most recently, when we had almost completed our experiments, Cocks *et al*. (1999) separately found that activation of PAR-1 as well as PAR-2 induces apamin-sensitive relaxation of the submaximally contracted strips of mouse gastric fundus and of guinea-pig taenia coli. This independent report is essentially consistent with our present findings in rat duodenal preparations. On the other hand, Nguyen *et al*. (1999) recently found that PAR-2 agonists activate charybdotoxin-sensitive potassium channels as well as chloride channels in dog pancreatic duct epithelial cells. Taken together with the present findings, PAR-1 and PAR-2 may

generally contribute to modulation of ion channels in various organs. Furthermore, surprisingly, our results that the relaxation due to PAR-1 activation was suppressed by combined administration of GF109203X and genistein, may suggest synergistic involvement of protein kinase C and tyrosine kinase, at least partially, in the PAR-1-mediated activation of apamin-sensitive potassium channels. This hypothesis may be supported by recent studies implying that tyrosine kinase is involved in the apamin-sensitive inhibitory effect of pituitary adenylate cyclase activating peptide on motility of the rat distal colon (Takeuchi *et al*., 1999) and that protein kinase C mediates metabolic stress-induced activation of apamin-sensitive potassium channels in human biliary cells and rat hepatoma cells (Wang *et al*., 1996; 1997).

In conclusion, PAR-1 appears to play a dual role in modulation of rat duodenal motility *via* activation of L-type calcium channels, protein kinase C, tyrosine kinase and calcium-activated, small-conductance potassium channels, and PAR-2 may play a minor role in facilitation of the duodenal motility through the same mechanisms as PAR-1.

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