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Proteinase-activated receptor-1 (PAR1) and PAR2 mediate relaxation of guinea pig internal anal sphincter

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ABSTRACT

Activation of proteinase-activated receptor-1 (PAR_1) and PAR_2 stimulates contraction of the rat but relaxation of the guinea pig colon. The aim of the present study was to investigate PAR effects on internal anal sphincter (IAS) motility. We measured relaxation of isolated muscle strips from the guinea pig IAS caused by PAR agonists using isometric transducers. Reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the existence of PAR. In the IAS, thrombin and PAR_1 peptide agonists TFLLR-NH_2 and SFLLRN-NH_2 evoked moderate to marked relaxation in a concentration-dependent manner. In addition, trypsin and PAR_2 peptide agonists 2-furoyl-LIGRLO-NH_2, SLIGRL-NH_2 and SLIGKV-NH_2 produced relaxation. In contrast, both PAR_1 and PAR_2 inactive control peptides did not elicit relaxation. Furthermore, the selective PAR_1 antagonist vorapaxar and PAR_2 antagonist GB 83 specifically inhibited thrombin and trypsin-induced relaxations, respectively. RT-PCR revealed the presence of PAR_1 and PAR_2 in the IAS. This indicates that PAR_1 and PAR_2 mediate the IAS relaxation. The relaxant responses of TFLLR-NH_2 and trypsin were attenuated by N(omega)-Nitro-L-arginine (L-NNA), indicating involvement of NO. These responses were not affected by tetrodotoxin, implying that the PAR effects are not neurally mediated. On the other hand, PAR_4 agonists GYPGKF-NH_2, GYPGQV-NH_2 and AYPGKF-NH_2 did not cause relaxation or contraction, suggesting that PAR_4 is not involved in the sphincter motility. Taken together, these results demonstrate that both PAR_1 and PAR_2 mediate relaxation of the guinea pig IAS through the NO pathway. PAR_1 and PAR_2 may regulate IAS tone and might be potential therapeutic targets for anal motility disorders.

1. Introduction

Proteinase-activated receptors (PARs) are a unique G protein-coupled receptor family activated by a variety of proteinases including thrombin and trypsin. Proteolysis of the PARs exposes receptor N-terminal sequences which act as activating ligands. Up to date, four subtypes of PARs, PAR_1, PAR_2, PAR_3, and PAR_4, have been described. The activating ligands are SFLLR, SLIGKV and GYPGQV for human PAR_1, PAR_2, and PAR_4, respectively. The PAR_3 peptide, TFRGAP, does not activate its receptor. Thrombin activates PAR_1 and PAR_4 whereas trypsin activates PAR_2. PARs are involved in aspects of both physiology and pathophysiology including inflammation disorders, cardiovascular diseases and cancer [1,2]. In the gastrointestinal system, PARs regulate gastrointestinal motility and secretion and may play important roles in colitis [2–4]. The gastrointestinal tract is exposed to trypsin, tryptase and bacterial proteinase, which can activate PARs [4,5]. Previous studies have demonstrated the effects of PAR on motility of the gastrointestinal and biliary tracts, including the esophagus [6,7], stomach [8–10], duodenum [11], gallbladder [12,13], common bile duct [14] and colon [8,15,16]. In the rat colon, activation of PAR_1, PAR_2 and PAR_4 stimulates contraction [15,16]. In contrast, PAR_1 and PAR_2 activation induces relaxation of the guinea pig colon [8]. In vivo stimulation of both PAR_1 and PAR_2 increases gastrointestinal transit in mice [17]. However, the PAR effects in the internal anal sphincter (IAS) are not known. We hypothesized that activation of PARs may alter IAS motility. The aim of the present study was to investigate the effects mediated by PARs in the guinea pig IAS in vitro. We found that activation of PAR_1 and PAR_2 but not PAR_4 promotes relaxation of the IAS.

2. Materials and methods

Thrombin (from bovine plasma), trypsin (from porcine pancreas, type IX-S), amastatin, N(omega)-Nitro-L-arginine (L-NNA), FSLLR-NH_2 (Phe-Ser-Leu-Leu-Arg-NH_2, inactive PAR_1 control peptide) and all buffer reagents were obtained from Sigma Chemical, St. Louis, MO, USA. TFLLR-NH_2 (Thr-Phe-Leu-Leu-Arg-NH_2, selective PAR_1 agonist), SFLLRN-NH_2 (Ser-Phe-Leu-Leu-Arg-Asn-NH_2, PAR_1 agonist), SLIGRL-NH_2 (Ser-Leu-Ile-Gly-Arg-Leu-NH_2, selective PAR_2 agonist), 2-furoyl-LIGRLO-NH_2 (2-furoyl-Leu-Ile-Gly-Arg-Leu-Orn-NH_2, selective PAR_2 agonist), GYPGQV-NH_2 (Gly-Tyr-Pro-Gly-Gln-Val-NH_2, selective PAR_4

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agonist), and AYPGKF-NH_2 (Ala-Tyr-Pro-Gly-Lys-Phe-NH_2, selective PAR_4 agonist) were obtained from American Peptide Company, Sunnyvale, CA, USA. SLIGKV-NH_2 (Ser-Leu-Ile-Gly-Lys-Val-NH_2, selective PAR_2 agonist), GYPGKF-NH_2 (Gly-Tyr-Pro-Gly-Lys-Phe-NH_2, selective PAR_4 agonist) and VKGILS-NH_2 (Val-Lys-Gly-Ile-Leu-Ser-NH_2, inactive PAR_2 control peptide) were purchased from Bachem, Bubendorf, Switzerland. Tetrodotoxin was obtained from Tocris, Cookson, Avonmouth Bristol, UK. Vorapaxar (SCH 530348) and GB 83 were purchased from Axon Medchem BV, Groningen, The Netherlands. RT-PCR reagents and primers for PAR_1 and PAR_2 were purchased from Invitrogen, Carlsbad, CA, and Integrated DNA Technologies, Inc., Coralville, IA, USA, respectively.

2.1. Measurement of relaxation of muscle strips isolated from guinea pig IAS

Male Hartley guinea pigs were obtained from the National Laboratory Animal Center, Taipei, Taiwan. Measurement of relaxation of muscle strips from the IAS was performed according to the procedure published previously [18,19] with modifications [20,21]. Male guinea pigs (350–450 g) were euthanized with CO_2. The anal canal was isolated, cut and placed in standard incubation solution, containing 118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 14 mM glucose, 1.2 mM NaH_2PO_4, and 1.8 mM $CaCl_2$, gassed with 95% O_2–5% CO_2. The final pH at 37 °C was 7.40 ± 0.05. The mucosa was removed. The isolated IAS muscle strip, 2 × 10 mm, was tied at both ends with silk sutures and placed in a 5-ml muscle bath containing the standard incubation solution (37 °C) continuously gassed with 95% O_2–5% CO_2. The strip was connected to isometric transducers (FT.03; Grass Technologies, West Warwick, RI, USA), which was connected to an integrated amplifier and computer recording system (BIOPAC Systems, Santa Barbara, CA, USA). The basal tension of the muscle strips was adjusted to 1.0 g. The experiment was started after a 45-min equilibration period. Only the IAS muscle strips that developed spontaneous tone were used. The spontaneous tone developed after the 45-min equilibration was 2.0 ± 0.1 g ($n = 20$).

PAR agonists were added to the muscle bath in a non-cumulative fashion, i.e. with single dose administration [6,7,20]. The relaxation responses were represented as a percentage (% papaverine) of the relaxation to 100 μM papaverine. Only one single dose response, with or without a receptor antagonist, tetrodotoxin or the NO synthase inhibitor, L-NNA, was studied with each preparation. For studies using receptor antagonists, tetrodotoxin and L-NNA, the muscle strips were exposed to the indicated concentrations of receptor antagonists, 1 μM tetrodotoxin and 1 mM L-NNA for 6 min, 15 min and 30 min, respectively, and then to PAR agonists. The muscle strips were exposed to 10 μM amastatin, a peptidase inhibitor, for 15 min, and then to the PAR peptide agonists to reduce peptide degradation [6,7,10]. In preliminary experiments, tetrodotoxin, L-NNA and amastatin did not affect basal tension of the IAS. All procedures were approved by the Institutional Animal Care and Use Committee of E-Da Hospital.

2.2. RT-PCR for detection of mRNA for PAR_1 and PAR_2 in guinea pig IAS

RT-PCR was performed for detection of mRNA for PAR_1, PAR_2 and β-actin in the guinea pig IAS as described previously [22,23] with modifications [14]. Total RNA was isolated from the guinea pig IAS using TRIzol reagent and treated with RNAse-free DNAse I. The Superscript II RNase H^- reverse transcriptase system was employed for the reverse transcription reaction. PCR amplification for PAR_1 was performed with Taq polymerase for 1 cycle at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 5 min. Similarly, PCR amplification for PAR_2 was performed with Taq polymerase for 1 cycle at 94 °C for 15 min, followed by 35 cycles at 94 °C for 30 s, 62 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min. PCR amplification for β-actin was performed with Taq polymerase for 1 cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 62 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 5 min. After amplification, the PCR products were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide and analyzed under UV light against DNA molecular markers. The PAR_1 and PAR_2 PCR products obtained were ligated into the pTZ57R/T vectors (Thermo Scientific, U.S.A.). The ligation mixture was used to transform *E. coli* strain Top10 to produce permanent clones for automated sequencing at Genomics BioSci & Tech (New Taipei City, Taiwan). The following primers were used:

PAR_1: 5′-TGTACGCCTCGGTCATGCTC-3′ and 5′-GACACAGACGCAGAGGAGGT-3′

PAR_2 [22,23]: 5′-CATGTTCAGCTACTTCCTCTCCTT-3′ and 5′-GGTTTTAACACTGGTGGAGCTTGA-3′

β-actin: 5′-CAAGGAGAAGCTATGCTATG-3′ and 5′-CAAGAAAGGGTGTAACGCAG-3′

The PAR_1 and β-actin primers were designed by Primer3 program and checked by NCBI BLAST for specificity.

2.3. Statistics

Results are expressed as means ± standard error of the mean (SEM). Dose–contraction curves were analyzed with the use of a nonlinear curve fitting program, GraphPad Prism, version 6, (La Jolla, CA, USA), to determine the half-maximal contraction (EC50) values. Statistical evaluation was performed using one-way analysis of variance (ANOVA) with the Dunnett post hoc procedure or Student's *t*-test when appropriate. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of thrombin and PAR_1 peptide agonists in IAS

In the guinea pig IAS strips, thrombin produced detectable relaxation at 1 μM. The highest concentration of thrombin tested (10 μM) caused a relaxant response of 41 ± 6% ($n = 4$) of 100 μM papaverine-induced relaxation (Figs. 1, 2). The PAR_1 peptide agonists TFLLR-NH_2 and SFLLRN-NH_2 caused marked whereas thrombin generated moderate, concentration-dependent relaxation (Figs. 1, 2). TFLLR-NH_2 caused detectable relaxation of IAS muscle strips at 1 μM. The highest concentration (30 μM) of TFLLR-NH_2 tested produced a relaxant response of 81 ± 4% ($n = 4$) of papaverine-induced relaxation (Figs. 1, 2). Similarly, SFLLRN-NH_2 caused detectable relaxation of the IAS muscle strips at 1 μM. The highest concentration (30 μM) of SFLLRN-NH_2 tested produced a relaxation of 81 ± 3% ($n = 3$) of papaverine-induced relaxation. In contrast, the PAR_1 inactive control peptide FSLLR-NH_2, at 30 μM, did not cause contraction or relaxation (Fig. 2). The TFLLR-NH_2-induced relaxation was not altered by 1 μM tetrodotoxin. Specifically, in the presence of 1 μM tetrodotoxin, 10 μM TFLLR-NH_2 produced 62 ± 9% ($n = 5$) papaverine-induced relaxation ($p > 0.05$, compared with TFLLR-NH_2 alone, which generated 74 ± 7%, $n = 9$, of papaverine-induced relaxation).

The relaxant response produced by TFLLR-NH_2 was attenuated by the NO synthase inhibitor L-NNA. In the presence of L-NNA 1 mM, 10 μM TFLLR-NH_2 produced a relaxant response of 38 ± 5% ($n = 4$) of papaverine-induced relaxation ($p = 0.007 < 0.05$, compared with TFLLR-NH_2 alone).

3.2. Effects of trypsin and PAR_2 peptide agonists in IAS

In the guinea pig IAS strips, trypsin caused marked, whereas the PAR_2 peptide agonists 2-furoyl-LIGRLO-NH_2, SLIGKV-NH_2 and SLIGRL-NH_2 produced mild to moderate relaxation of the guinea pig IAS in a concentration-dependent manner (Figs. 1, 3). Trypsin caused detectable relaxation at 300 nM, half-maximal relaxation at 520 ± 50 nM and maximal relaxation at 100 μM, which evoked a relaxant response of 83 ± 1% ($n = 4$) of 100 μM papaverine-induced relaxation (Fig. 3). 2-Furoyl-

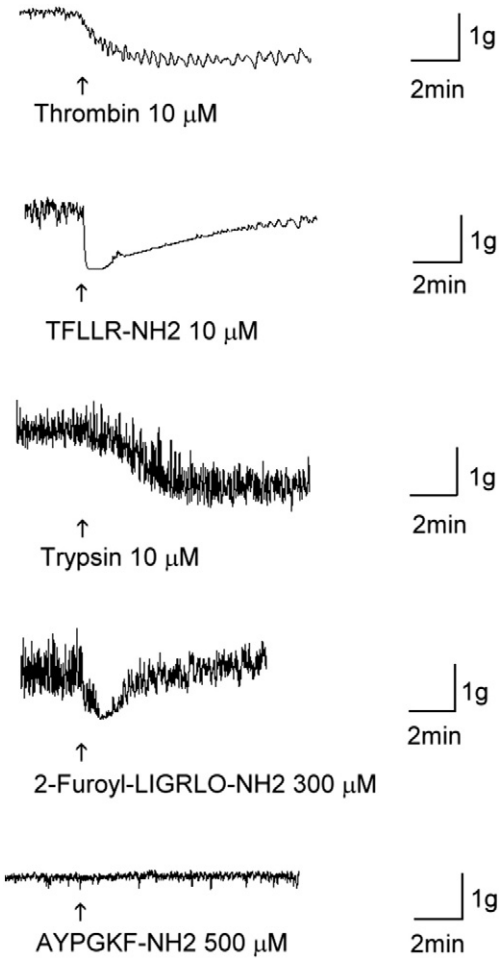

Fig. 1. Typical tracings showing relaxations of the guinea pig internal anal sphincter induced by thrombin and PAR_1 selective agonist TFLLR-NH_2 (Thr-Phe-Leu-Leu-Arg-NH2), trypsin and PAR_2 selective agonist 2-furoyl-LIGRLO-NH_2 (2-furoyl-Leu-Ile-Gly-Arg-Leu-Orn-NH2) but not PAR_4 selective agonist AYPGKF-NH_2 (Ala-Tyr-Pro-Gly-Lys-Phe-NH2).

LIGRLO-NH_2 was much less potent than trypsin and generated detectable relaxation at 30 μM. The highest concentration (300 μM) of 2-furoyl-LIGRLO-NH_2 tested produced a relaxant response of 58 ± 9% ($n = 4$) of papaverine-induced relaxation (Fig. 3). SLIGRL-NH_2 caused detectable relaxation at 10 μM. The highest concentration (300 μM) of SLIGRL-NH_2 tested generated a relaxant response of 23 ± 8% ($n = 3$) of papaverine-induced relaxation (Fig. 3). Similarly, SLIGKV-NH_2 caused

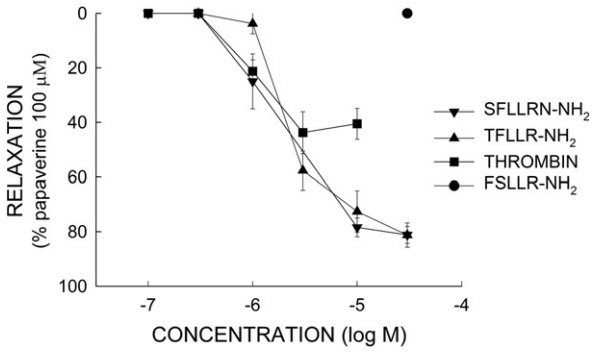

Fig. 2. Ability of thrombin and PAR_1 peptide agonists TFLLR-NH_2 (Thr-Phe-Leu-Leu-Arg-NH2) and SFLLRN-NH_2 (Ser-Phe-Leu-Leu-Arg-Asn-NH2) to cause relaxation of the guinea pig internal anal sphincter. The PAR_1 inactive control peptide FSLLR-NH_2 (Phe-Ser-Leu-Leu-Arg-NH_2) did not cause relaxation. Values are expressed as percent of 100 μM papaverine-induced relaxation. Results given are from at least three experiments. Vertical bars represent ± standard error of the mean (SEM).

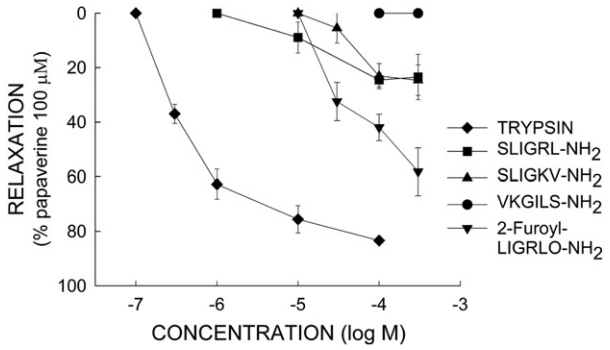

Fig. 3. Ability of trypsin and PAR_2 selective agonists 2-furoyl-LIGRLO-NH_2 (2-furoyl-Leu-Ile-Gly-Arg-Leu-Orn-NH_2), SLIGKV-NH_2 (Ser-Leu-Ile-Gly-Lys-Val-NH_2) and SLIGRL-NH2 (Ser-Leu-Ile-Gly-Arg-Leu-NH_2) to cause relaxation of the guinea pig internal anal sphincter. The inactive PAR_2 control peptide VKGILS-NH_2 (Val-Lys-Gly-Ile-Leu-Ser-NH_2) did not cause relaxation. Values are expressed as percent of 100 μM papaverine-induced relaxation. Results given are from at least three experiments. Vertical bars represent ± standard error of the mean (SEM).

detectable relaxation at 30 μM. The highest concentration (300 μM) of SLIGKV-NH_2 tested produced a relaxant response of 25 ± 6% ($n = 3$) of papaverine-induced relaxation (Fig. 3). In contrast, the PAR_2 inactive control peptide VKGILS-NH_2 did not cause contraction or relaxation when tested at concentrations up to 300 μM (Fig. 3). The trypsin-evoked relaxation was not altered by tetrodotoxin. Specifically, in the presence of 1 μM tetrodotoxin, trypsin (10 μM) produced a relaxant response of 72 ± 5% ($n = 5$) of papaverine-induced relaxation ($p > 0.05$, compared with trypsin alone, which evoked a relaxant response 76 ± 5% ($n = 6$) of papaverine-induced relaxation).

The relaxant response produced by trypsin was attenuated by L-NNA. In the presence of 1 mM L-NNA, 1 μM trypsin produced a relaxant response of 30 ± 3% ($n = 4$) of papaverine-induced relaxation ($p < 0.001$, compared with trypsin alone, 63 ± 5%, $n = 4$).

3.3. No effects of PAR_4 agonists on IAS contraction or relaxation

The PAR_4 peptide agonists AYPGKF-NH_2 (Fig. 1), GYPGKF-NH_2 and GYPGQV-NH_2 (data not shown), at concentrations up to 500 μM, did not cause contraction or relaxation of the guinea pig IAS strips.

3.4. Effects of PAR_1 and PAR_2 antagonists on thrombin or trypsin-induced IAS relaxation

The selective PAR_1 antagonist vorapaxar (3 μM) and PAR_2 antagonist GB 83 (3 μM) alone did not cause contraction or relaxation (data not shown). In the IAS, vorapaxar abolished whereas GB 83 did not alter the thrombin response (Fig. 4). Specifically, the relaxation caused by 3 μM thrombin alone was 44 ± 8% ($n = 4$) of papaverine-induced relaxation, whereas the relaxations caused by thrombin with 3 μM vorapaxar and 3 μM GB 83 were 0 ± 0% ($n = 5$) and 47 ± 6% ($n = 5$), respectively ($p < 0.001$ and $p = 0.79$, respectively, compared with thrombin alone using ANOVA; Fig. 4). Furthermore, GB 83 markedly inhibited whereas vorapaxar did not alter the trypsin response significantly. Specifically, the relaxation caused by 1 μM trypsin alone was 63 ± 5% ($n = 5$) of papaverine-induced relaxation, whereas the relaxations caused by trypsin with 3 μM GB 83 and 3 μM vorapaxar were 15 ± 9% ($n = 4$) and 46 ± 6% ($n = 4$), respectively ($p < 0.001$ and $p = 0.09$, respectively, compared with trypsin alone using ANOVA; Fig. 4).

3.5. PAR_1 and PAR_2 expression in IAS

RT-PCR was performed to examine the mRNA expression of PAR_1, PAR_2, and β-actin in the guinea pig IAS. As shown in Fig. 5, amplification of the cDNA yielded 521, 472, and 532 base-pair products for PAR_1, PAR_2, and β-actin, respectively, as predicted [22,23]. Sequencing of the

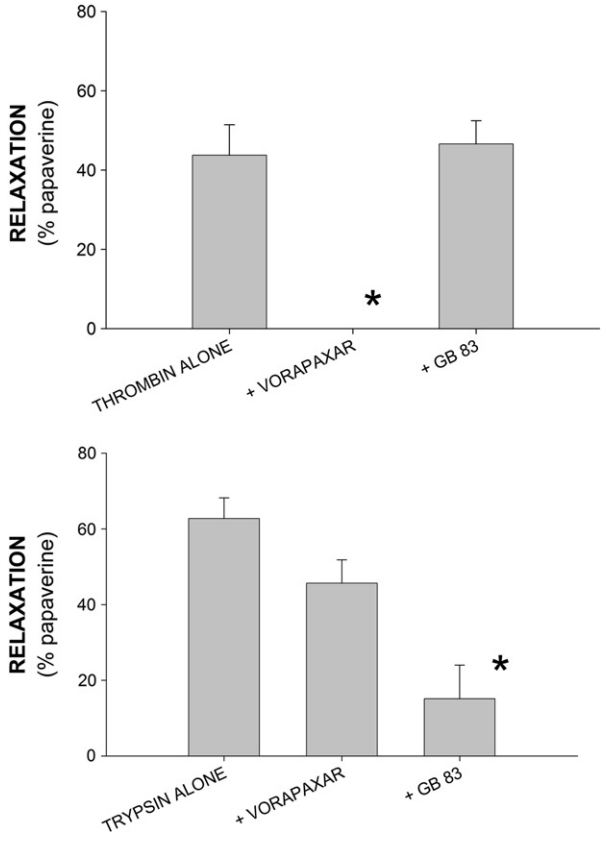

Fig. 4. Ability of PAR_1 antagonist vorapaxar and PAR_2 antagonist GB 83 to inhibit thrombin (upper panel) or trypsin (lower panel)-induced relaxation of the guinea pig internal anal sphincter. Values are expressed as percent of 100 μM papaverine-induced relaxation. Results given are from at least four experiments. Vertical bars represent ± standard error of the mean (SEM).

PAR_1 and PAR_2 RT-PCR products and blasting against the NCBI nucleotide database confirmed that they represented partial sequences of guinea pig PAR_1 and PAR_2 (Supplement Fig. 1).

4. Discussion

PAR activation modulates gastrointestinal motility from the esophagus to colon. The present study demonstrates that PAR_1 and PAR_2 activation can also relax the IAS. In addition, this study unveils that NO is involved in both PAR_1 and PAR_2-mediated relaxation of the guinea pig IAS, similar to the guinea pig lower esophageal sphincter [7].

In the isolated IAS strips, thrombin and the PAR_1 peptide agonists TFLLR-NH_2 and SFLLRN-NH_2 as well as trypsin and the PAR_2 peptide agonists 2-furoyl-LIGRLO-NH_2, SLIGRL-NH_2 and SLIGKV-NH_2 evoked concentration-dependent relaxation. Furthermore, the PAR_1 antagonist vorapaxar and PAR_2 antagonist GB 83 specifically inhibited the thrombin and trypsin responses, respectively. In addition, RT-PCR identified

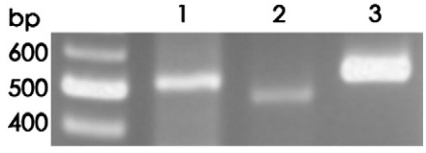

Fig. 5. Reverse transcription polymerase chain reaction (RT-PCR) analyses of the expression of PAR_1, PAR_2, and β-actin mRNA in the guinea pig internal anal sphincter. Total RNA was extracted from the internal anal sphincter, reverse transcribed, and amplified using PAR_1, PAR_2 or β-actin-specific primers. The amplified products were electrophoresed on an agarose gel. Results presented are representative of three experiments. Lane 1, PAR_1; Lane 2, PAR_2; Lane 3, β-actin.

the expression of PAR_1 and PAR_2 in the guinea pig IAS. This indicates that both PAR_1 and PAR_2 mediate relaxation in the guinea pig IAS.

The PAR_1 and PAR_2-mediated IAS relaxation might involve NO as the TFLLR-NH_2 and trypsin-induced relaxant responses were attenuated by the NO synthase inhibitor L-NNA. This is in agreement with previous studies showing that NO is one of the major signaling pathways for relaxation in the IAS [24]. All three PAR_4 peptide agonists, AYPGKF-NH_2, GYPGKF-NH_2 and GYPGQV-NH_2, exhibited no contraction or relaxation in IAS strips, suggesting that PAR_4 activation does not affect IAS motility. Previously, PAR_4 agonists also were found to have no motility effects in the esophagus, duodenum and gallbladder [6,7,11–13]. However, PAR_4 agonists were reported to elicit contraction of the colon and relaxation of the common bile duct [14,16].

In the gastrointestinal tract, PAR_1 and PAR_2 stimulate contraction of the human esophagus [6], guinea pig and rat stomach [9,10], human and guinea pig gallbladder [12,13] as well as rat colon [15]. In addition, PAR_1 and PAR_2 activation evokes relaxation of the guinea pig lower esophageal sphincter [7] and guinea pig colon [8]. Both PAR_1 and PAR_2 elicit a dual action, i.e., relaxation followed by contraction, in the mouse gastric fundus [8] and rat duodenum [11]. Furthermore, the results in the present study showed that both PAR_1 and PAR_2 mediate relaxation of the guinea pig IAS. Thus, PAR_1 and PAR_2 mediate similar motility effects and are the predominant PAR subtypes regulating gastrointestinal motility, while PAR_4 has minor impact in this aspect. The IAS basal tone is myogenic but altered by external neurohumoral signals [24]. Previous studies have shown that IAS relaxations are mediated by β-adrenoceptors, muscarinic, purinergic, endothelin and angiotensin AT2 receptors [19,25–28]. In contrast, stimulations of α-adrenoceptors and angiotensin AT1 receptors result in contractions [25,26,29]. The present study showed that PAR_1 and PAR_2 mediate relaxation in the guinea pig IAS under physiological conditions ex vivo. PAR_1 and PAR_2 might regulate the basal tone of IAS, which plays an important role in maintaining the anal continence. Previous studies strongly suggested that PAR_1 and PAR_2 are involved in the pathogenesis of colitis [2]. Whether PAR_1 and PAR_2 are involved in the pathogenesis of anorectal motility disorders is not known. Potent PAR agonists and antagonists are being developed recently [2]. Further studies using these agonists and antagonists are warranted to investigate the involvement of PARs in anorectal motility disorders. Currently, glyceryl trinitrate and the calcium channel blocker nifedipene are commonly used for topical treatment of chronic anal fissure. However, there is a moderate incidence of side effects and the response is inadequate in some patients [30]. Our findings may help the development of new therapeutic agents to target the IAS. PAR_1 and PAR_2 agonists, which can relax IAS, are of potential therapeutic importance in chronic anal fissure or anal pain after hemorrhoidectomy [25,30–32]. Studies on PAR effects in the human IAS are needed to investigate these potential applications [33].

In conclusion, these results provide evidence that both PAR_1 and PAR_2 mediate guinea pig IAS relaxation through the NO pathway. PAR_1 and PAR_2 may regulate IAS tone and might be potential therapeutic targets for anal motility disorders including anal fissure.

Conflict of interest

The author declares that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.regpep.2014.03.001.

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