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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.

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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-

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