Effects of trypsin, thrombin and proteinase-activated receptors on guinea pig common bile duct motility

Shih-Che Huang *

Department of Internal Medicine, E-Da Hospital, and School of Chinese Medicine for Post-Baccalaureate, I-Shou University, Kaohsiung 824, Taiwan

A B S T R A C T

Trypsin and thrombin activate proteinase-activated receptors (PARs), which modulate gastrointestinal motility. The common bile duct is exposed to many proteinases that can activate PARs, especially during infection and stone obstruction. We investigated PAR effects on common bile duct motility in vitro. Contraction and relaxation of isolated guinea pig common bile duct strips caused by PAR1, PAR2, PAR3, and PAR4 agonists were measured using isometric transducers. Reverse transcription polymerase chain reaction (RT-PCR) was performed to determine the expression of PAR1, PAR2, PAR3, and PAR4 peptide agonists, TFLLR-NH2 and SFLRN-NH2, evoked moderate relaxation of the carbachol-contracted common bile duct in a concentration-dependent manner. Trypsin and three PAR2 peptide agonists, 2-furoyl-LIGRLO-NH2, SLIGKV-NH2, and SLIGRL-NH2, generated moderate to marked relaxation as well. The existence of PAR1, PAR2, PAR3 mRNA in the common bile duct was identified by RT-PCR. Moreover, two PAR2-selective agonists, AYPGKF-NH2 and GYPQGV-NH2, produced relaxation of the common bile duct. In contrast, all PAR1, PAR2, PAR3 inactive control peptides did not elicit relaxation. This indicates that PAR1, PAR2, and PAR3 mediate common bile duct relaxation. The thrombin, TFLLR-NH2, trypsin, and AYPGKF-NH2-induced responses were not affected by tetrodotoxin, implying that the PAR effects are not neural- ly mediated. Our findings provide the first evidence that PAR1 and PAR2 mediate whereas agonists of PAR4 elicit relaxation of the guinea pig common bile duct. Trypsin and thrombin relax the common bile duct. PARs may play an important role in the control of common bile duct motility.

1. Introduction

Trypsin and thrombin activate proteinase-activated receptors (PARs) through proteolysis. The proteolysis of N-terminals of PARs by proteinases unmasks new terminal sequences which activate PARs [1–5]. Four subtypes of PARs, i.e. PAR1, PAR2, PAR3, and PAR4, have been described. The tethered peptide ligands are SFLLR, SLIGKV, and GYPQGV for human, as well as SFFLR, SLIGRL, and GYPQGF for mouse PAR1, PAR2, and PAR4, respectively. On the other hand, the ability of the PAR3 tethered peptide, TFRGAP, to stimulate PAR3 is unclear [1]. Thrombin is a main activator of PAR1, PAR2, and PAR4 and trypsin, of PAR1 [1–4]. PARs are found in various tissues, including the gastrointestinal tract [1–4,6]. PAR1, PAR2, and PAR4 modulate the gastrointestinal motility and secretion [1,6–14]. In the human and guinea pig gallbladder, both PAR1 and PAR2 mediate contraction; however, PAR4 activation does not alter gallbladder motility [10,11]. The PAR effects on the common bile duct (CBD) motility are not known. We hypothesized that PAR activation might cause contraction or relaxation of the CBD, similar to the gallbladder. The CBD is exposed to many proteinases that can activate PARs, especially during CBD infection and stone obstruction. Pancreatic trypsin, extravasated plasma thrombin and bacterial proteinases may activate PARs [3,6,10]. Because CBD motility could affect bile flow, stasis of which may promote the formation of CBD stone [15,16], it is important to study the effects of PARs on CBD motility. The purpose of the present study was to investigate the effects mediated by PAR1, PAR2, and PAR4 on CBD motility. We found that activation of PAR advocates relaxation but not contraction of the CBD, which is the opposite of the PAR effects in the gallbladder.

2. Materials and methods

Male Hartley guinea pigs (300–350 g) were obtained from the National Laboratory Animal Center, Taiwan. All procedures were performed in compliance with relevant laws and institutional guidelines and approved by the Institutional Animal Care and Use Committee of E-Da Hospital. Thrombin (from bovine plasma), trypsin (from porcine pancreas, type IX-S), amastatin, carbachol, and all buffer reagents were purchased from Sigma Chemical, St Louis, MO, USA; TFLLR-NH2, (Thr-Phe-Leu-Leu-Arg-NH2, selective PAR1 agonist), SFLRN-NH2, (Ser-Phe-Leu-Leu-Arg-Asn-NH2, PAR1 agonist), RLLFT-NH2, (Arg-Leu-Leu-Phe-Thr-NH2, inactive PAR1 control peptide), SLIGRL-NH2, (Ser-Leu-Ile-Gly-Arg-Leu-NH2, selective PAR2 agonist), 2-furoyl-LIGRLO-NH2, (2-furoyl-Leu-Ile-Gly-Arg-Leu-Orn-NH2, selective PAR2 agonist),

* E-Da Hospital, 1, Yi-Da Road, Yan-Chau, Kaohsiung 824, Taiwan. Tel.: +886 7 615 0011 x32981; fax: +886 7 615 0940.

E-mail addresses: shihchehuang@hotmail.com, huangshihche@gmail.com.
GYPGQV-NH₂ (Gly-Tyr-Pro-Gly-Gln-Val-NH₂, selective PAR1 agonist), and AYPKCFK-NH₂ (Ala-Tyr-Pro-Gly-Lys-Phe-NH₂, selective PAR2 agonist) were obtained from American Peptide Company, Sunnyvale, CA, USA. SIGKLV-NH₂ (Ser-Leu-Ile-Gly-Lys-Val-NH₂, selective PAR2 agonist) and VKGILS-NH₂ (Val-Lys-Gly-Ile-Leu-Ser-NH₂, inactive PAR2 control peptide) were purchased from Bachem, Bubendorf, Switzerland. YAPGKF-NH₂ (Tyr-Ala-Pro-Gly-Lys-Phe-NH₂, inactive PAR2 control peptide) was purchased from Peptides International, Louisville, Kentucky, USA. Tetrodotoxin was obtained from Toctis, Cookson, Avonmouth Bristol, UK. Reverse transcription polymerase chain reaction (RT-PCR) reagents and primers for guinea pig PAR1 and PAR2 were purchased from Invitrogen, Carlsbad, CA, and Integrated DNA Technologies, Inc., Coralville, IA, USA, respectively.

2.1. Measurement of contraction and relaxation of guinea pig CBD muscle strips

Measurements of contraction and relaxation of isolated muscle strips from the guinea pig CBD were performed according to the procedure described previously [17–19] with minor modifications [20]. In brief, the guinea pig was euthanized by CO₂, PAR script II RNase H using TRIzol reagent and treated with RNAse-free DNAse I. The superfused strips from the guinea pig CBD were performed according to the procedure. Products were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide and analyzed under UV light against DNA molecular markers. The following primers were used [9,21,22]:

PAR₁: Forward 5′-CCGCTCATTTTTTCTCAGGAA-3′
Reverse 5′-AATCGGTCGCCGAGAAGT-3′
PAR₂: Forward 5′-CATGTCAGCTTCTCCTGTTTT-3′
Reverse 5′-AATCGGGTCCGGAGAAGT-3′

2.2. RT-PCR for detection of mRNA for PAR₁ and PAR₂ in guinea pig CBD

RT-PCR for detection of mRNA for PAR1 and PAR2 in the guinea pig CBD was performed as described previously [9,21,22] with minor modification [7]. Total RNA was isolated from the guinea pig CBD using TRIzol reagent and treated with RNase-free DNase I. The super-script II RNase H reverse transcriptase system was employed for reverse transcription. Polymerase chain reaction amplification for PAR1 was performed with Taq polymerase at 94 °C for 5 min, followed by 45 cycles at 94 °C for 30 s, 56 °C for 15 s, 72 °C for 30 s, and, finally 72 °C for 5 min. Polymerase chain reaction amplification for PAR2 was performed with Taq polymerase 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. 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 following primers were used [9,21,22]:

PAR₁: Forward 5′-CCGCTCATTTTTTCTCAGGAA-3′
Reverse 5′-AATCGGTCGCCGAGAAGT-3′
PAR₂: Forward 5′-CATGTCAGCTTCTCCTGTTTT-3′
Reverse 5′-AATCGGGTCCGGAGAAGT-3′

2.3. Analysis of data

Results are expressed as means±standard error of the mean (SEM). Statistical evaluation was performed using Student’s t-test or one-way analysis of variance (ANOVA), corrected by the Dunnett procedure. P<0.05 was considered statistically significant.

3. Results

3.1. Effects of thrombin and PAR₁ peptide agonists in CBD

In the resting guinea pig CBD strips, thrombin, 30 μM, did not cause contraction or relaxation. We then evaluated the ability of thrombin to evoke relaxation of carbachol pre-contracted CBD strips. Carbachol (1 μM) increased the force of the CBD strips by 0.36±0.03 g (n=15) and this contraction reached a plateau within 6 min (Fig. 1). Adding thrombin to the carbachol-contracted muscle strips at the plateau evoked a moderate, sustained and concentration-dependent relaxation (Figs. 1, 2). Thrombin caused detectable relaxation of the carbachol-contracted CBD strips at 3 μM and maximal relaxation at 30 μM, which produced a 41±1% papaverine (100 μM)-induced relaxation (Fig. 2). Similarly, adding TFLLR-NH₂ and SFLLRN-NH₂ to the carbachol-contracted muscle strips at the plateau resulted in a moderate and concentration-dependent relaxation (Figs. 1, 2). TFLLR-NH₂ produced detectable relaxation of the carbachol-contracted strips at 10 μM and maximal relaxation at 30 μM, which produced a 38±2% papaverine-induced relaxation. The highest concentration (100 μM) of TFLLR-NH₂ tested produced a 37±1% papaverine-induced relaxation of carbachol-contracted CBD (Fig. 2). SFLLRN-NH₂ caused detectable relaxation of the carbachol-contracted strips at 30 μM. The highest concentration (300 μM) of SFLLRN-NH₂ tested produced a 45±5% papaverine-induced relaxation of carbachol-contracted CBD (Fig. 2). In contrast, 100 μM RLLFT-NH₂, the PAR₁ inactive control peptide [23], did not cause relaxation in carbachol-contracted CBD strips (Fig. 2). The thrombin and TFLLR-NH₂-induced relaxations were not altered by

Fig. 1. Typical tracings showing the relaxation of carbachol-contracted guinea pig common bile duct with the addition of thrombin (upper panel) and the PAR₁ selective agonist TFLLR-NH₂ (Thr-Phe-Leu-Arg-NH₂, lower panel).
Values are expressed as percent of papaverine (100 μM)-induced relaxation. Results are given from at least three experiments. Vertical bars represent ± standard error of the mean (SEM).

1 mM tetrodotoxin. Specifically, in the presence of 1 mM tetrodotoxin, thrombin (30 μM) generated a 42 ± 7% papaverine-induced relaxation (P > 0.05, compared with thrombin alone) and TFLRN-NH$_2$ (30 μM) produced a 47 ± 3% papaverine-induced relaxation (P > 0.05, compared with TFLRN-NH$_2$ alone).

3.2. Effects of trypsin and PAR$_2$ peptide agonists in CBD

Adding trypsin (10 μM) and 2-furoyl-LIGRLO-NH$_2$ (300 μM) to the resting CBD muscle strips elicited a 65 ± 11% and 61 ± 5% papaverine (100 μM)-induced relaxation, respectively. To further characterize the PAR$_2$ mediates the CBD relaxation, the ability of trypsin, 2-furoyl-LIGRLO-NH$_2$, SLIGKV-NH$_2$ and SLIGRL-NH$_2$ to generate relaxation of carbachol pre-contracted guinea pig CBD strips was determined. Adding trypsin to the carbachol-contracted muscle strips evoked a marked, sustained and concentration-dependent relaxation (Figs. 3, 4). Trypsin caused detectable relaxation of the carbachol-contracted strips at 300 nM and maximal relaxation at 10 μM, which caused a 68 ± 9% papaverine (100 μM)-induced relaxation of the carbachol-induced CBD. The highest concentration (100 μM) of trypsin tested produced a 69 ± 5% papaverine-induced relaxation of carbachol-contracted CBD (Fig. 4). Furthermore, adding 2-furoyl-LIGRLO-NH$_2$, SLIGRL-NH$_2$ and SLIGKV-NH$_2$ to the carbachol-contracted muscle strips induced moderate and concentration-dependent relaxation (Fig. 4). The selective PAR$_2$ agonist 2-furoyl-LIGRLO-NH$_2$ produced detectable relaxation of the carbachol-contracted strips at 10 μM. The highest concentration (300 μM) of 2-furoyl-LIGRLO-NH$_2$ tested evoked a 55 ± 11% papaverine-Ainduced relaxation. Similarly, SLIGRL-NH$_2$ generated detectable relaxation of the carbachol-contracted strips at 10 μM. The highest concentration (300 μM) of SLIGRL-NH$_2$ tested produced a 46 ± 3% papaverine-induced relaxation. Furthermore, SLIGKV-NH$_2$ caused detectable relaxation of the carbachol-contracted strips at 10 μM. The highest concentration (100 μM) of SLIGKV-NH$_2$ tested produced a 41 ± 10% papaverine-induced relaxation (Fig. 4).

2-furoyl-LIGRLO-NH$_2$ generated a 31 ± 6% papaverine-induced relaxation of the carbachol-contracted strips at 300 μM. The highest concentration (500 μM) of GYPGVQV-NH$_2$ generated a 44 ± 10% papaverine-induced relaxation (Fig. 6). In contrast, the PAR$_2$ control peptide, YAPGKF-NH$_2$ [24], did not cause relaxation at 300 μM (Fig. 6). Furthermore, the AYPGKF-NH$_2$ induction was not altered by tetrodotoxin. In the presence of 1 mM tetrodotoxin, AYPGKF-NH$_2$ (300 μM) produced a 36 ± 8% papaverine-induced relaxation (P > 0.05, compared with AYPGKF-NH$_2$ alone).

3.3. Effects of PAR$_2$ peptide agonists in CBD

Two PAR$_2$ peptide agonists, AYPGKF-NH$_2$ and GYPGVQV-NH$_2$, evoked moderate relaxation in carbachol (1 μM)-contracted guinea pig CBD strips (Figs. 5, 6). AYPGKF-NH$_2$ caused detectable and a 37 ± 1% papaverine-induced relaxation of the carbachol-contracted strips at 100 μM and 300 μM, respectively. The highest concentration (500 μM) of AYPGKF-NH$_2$ tested produced a 44 ± 10% papaverine-induced relaxation. Similarly, GYPGVQV-NH$_2$ generated a 31 ± 6% papaverine-induced relaxation of the carbachol-contracted strips at 300 μM. The highest concentration (500 μM) of GYPGVQV-NH$_2$ tested yielded a 41 ± 11% papaverine-induced relaxation (Fig. 6). In contrast, the PAR$_2$ control peptide, YAPGKF-NH$_2$ [24], did not cause relaxation at 300 μM (Fig. 6). Furthermore, the AYPGKF-NH$_2$ induction was not altered by tetrodotoxin. In the presence of 1 mM tetrodotoxin, AYPGKF-NH$_2$ (300 μM) produced a 36 ± 8% papaverine-induced relaxation (P > 0.05, compared with AYPGKF-NH$_2$ alone).
3.4. PAR₁ and PAR₂ expression in CBD

RT-PCR was used to examine mRNA expression of PAR₁ and PAR₂ in the guinea pig CBD. As shown in Fig. 7, amplification of the cDNA yielded 393 and 472 base-pair products for PAR₁ and PAR₂, respectively, as predicted [9,21,22]. No analysis of PAR₃ expression in the CBD was performed because the gene sequence of guinea pig PAR₃ was not identified (Pubmed search).

4. Discussion

Alteration in CBD motility plays an important role in pathological conditions including common bile stone [15,16]. Nevertheless, little is known about the nervous or hormonal control of the CBD motility. Carbachol, cholecystokinin, tachykinin and endothelin have been described to stimulate contraction of the CBD [17,18,25–27]. In the present work, we have shown for the first time that trypsin and thrombin relax the CBD. In addition, the present study provides evidence that PAR₁, PAR₂ and PAR₃ mediate relaxation of the CBD.

In carbachol-contracted guinea pig CBD strips, the PAR₁ agonists thrombin, TFLLR-NH₂ and SFLRR-NH₂ as well as PAR₂ agonists trypsin, 2-furoyl-LIGRLO-NH₂, SLIGRL-NH₂ and SLIGKV-NH₂ generated moderate to marked relaxation in a concentration-dependent manner. In contrast, both PAR₁ and PAR₂ inactive control peptides did not produce any relaxation. The existence of PAR₁ and PAR₂ in the guinea pig CBD was confirmed by RT-PCR. This indicates that both PAR₁ and PAR₂ mediate CBD relaxation, different to the PAR₃ and PAR₄-mediated gallbladder contraction [10,11]. Trypsin and the PAR₂ selective agonist 2-furoyl-LIGRLO-NH₂ were more efficacious than thrombin and the PAR₁ selective agonist TFLLR-NH₂. The reason for the greater PAR₂ effect is unclear and may include differences between PAR₁ and PAR₂ signaling pathways [1].

In previous studies, the PAR₁ peptide agonists exhibited contractile effects in the colon but no effects in the duodenum and gallbladder [10,11,13,14]. Our findings demonstrate that PAR₄ is involved in CBD motility modulation. Both PAR₄-selective agonists, AYPGKF-NH₂ and GYPGQV-NH₂, but not the inactive control peptide evoked relaxation in the carbachol-contracted CBD strips. This indicates that PAR₄ mediates relaxation in the CBD. The relaxant effect of PAR₄ is similar to that of PAR₁ and PAR₂ in the CBD. Unlike PAR₁ and PAR₂, PAR₄ does not mediate contraction or relaxation in the gallbladder and lower esophageal sphincter [10,11,20].

Similar to the PAR-mediated lower esophageal sphincter relaxation and gallbladder contraction, which were tetrodotoxin-insensitive [10,11,20], the relaxant responses of thrombin, TFLLR-NH₂, trypsin and AYPGKF-NH₂ in the CBD were not affected by tetrodotoxin. This suggests that neural mechanisms are probably not involved.

Bile stasis and infection are risk factors of CBD stone [15,16,28]. The CBD is exposed to many proteinases during infection, stone obstruction or stricture. Further study is warranted to explore the involvement of PAR activation in the pathogenesis of cholangitis or CBD stone. PAR-related agents may affect CBD function and might be potential therapeutic agents for biliary motility disorders. PAR₁ and PAR₂ agonists may contract the gallbladder and relax the CBD, whereas PAR₄ antagonists may inhibit bile duct relaxation without affecting gallbladder motility.

Taken together, our findings provide the first evidence that PAR₁ and PAR₂ mediate whereas agonists of PAR₄ elicit relaxation of the guinea pig CBD. Trypsin and thrombin cause CBD relaxation. PARs may play an important role in the control of CBD motility.

Conflict of interest statement

The author declares that there are no conflicts of interest.

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