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Trypsin-induced biphasic regulation of tone in the porcine lower esophageal sphincter

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ABSTRACT

The lower esophageal sphincter (LES) plays an important role in coordinated esophageal motility. The present study aimed to elucidate how trypsin affects LES contractility. Porcine LES circular smooth muscle strips were prepared. Contractile responses to trypsin were assessed. Trypsin (300 nM) induced a transient contraction. At concentrations of 1 μM or higher, trypsin induced biphasic responses, consisting of a transient contraction followed by a transient relaxation. Pretreatment with either 1 μM tetrodotoxin or carbenoxolone had no effect on these responses. In contrast, trypsin-induced responses were completely blocked by pretreatment with the serine protease inhibitor. Pretreatment with 10 μM FSLLRY-NH_2, a PAR_2 antagonist, significantly inhibited trypsin-induced biphasic responses. Trypsin (1 μM)-induced contractions were partially inhibited by pretreatment with 10 μM Y-27632. In addition, trypsin (10 μM)-induced relaxation was partially inhibited by pretreatment with 10 μM Y-27632, 10 μM PD98059 or 10 μM SB203580. Trypsin-induced relaxation was abolished by increasing the extracellular K^+ concentration to 40 mM, but not by pretreatment with L-arginine methyl ester. Furthermore, trypsin-induced relaxation was partially inhibited by pretreatment with 10 μM glibenclamide or 1 μM 4-aminopyridine. Trypsin causes biphasic regulation of LES tone by directly acting on smooth muscle. Rho-associated protein kinase (ROK) is involved in trypsin-induced contraction, whereas ROK, ERK1/2, p38MAPK, and membrane hyperpolarization are involved in relaxation. The regulation of LES tone by trypsin may play a role in esophageal motility.

1. Introduction

Trypsin exerts various cellular effects by activating proteinase-activated receptors (PARs), a unique family of G protein-coupled receptors (Cocks et al., 1999). Among the four PAR subtypes, PAR_1, PAR_2 and PAR_4 are potential receptors for trypsin (Cocks et al., 1999). PARs are expressed in smooth muscle cells and/or their adjacent cells throughout the gastrointestinal tract and PARs modulate the contractility of gastrointestinal smooth muscle (Kawabata et al., 2008). The Ca^{2+}-dependent phosphorylation of the regulatory light chain of myosin II plays a fundamental role in the contraction of smooth muscle (Pfitzer, 2001). Trypsin activation of PAR_2 induces Ca^{2+} signaling and activation of several protein kinases, including protein kinase C (PKC), Rho-associated protein kinase (ROK), extracellular signal-activated protein kinase (ERK) 1/2 and p38 MAP kinase (p38MAPK) (Adams et al., 2011). All of these signals conceivably contribute to the effect of trypsin on gastrointestinal smooth muscle contraction. PKC, ROK, ERK1/2 and p38MAPK are known to inhibit the activity of myosin light chain phosphatase, thus contributing to smooth muscle contraction (Eto et al., 1995; Ihara et al., 2007; Kimura et al., 1996). Regulation of gastrointestinal contractility by trypsin/PARs has been reported in stomach, small intestine and colon (Cocks et al., 1999; Kawabata et al., 1999; Mule et al., 2002). However, limited information is available regarding the regulatory role of trypsin in the function of esophagus.

Abbreviations: LES, lower esophageal sphincter; GERD, gastro-esophageal reflux disease; ROK, Rho-associated protein kinase; PAR, proteinase-activated receptor; PKC, protein kinase C; ERK, extracellulular signal-activated protein kinase; p38MAPK, p38 MAP kinase; NES, normal extracellular solution; 118-KES, 118 mM K^+ extracellular solution; p-APMSF, p-amidinophenyl methanesulfonyl fluoride; AP, activating peptide; L-NAME, N^{ω}-nitro-L-arginine methyl ester

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It has been reported that the level of PAR_2 expression is up-regulated in the esophageal mucosa of patients with gastro-esophageal reflux disease (GERD) and correlates with pathological characteristics of GERD (Kandulski et al., 2010). The activation of PAR_2 by trypsin induces the secretion of interleukin 8, which may contribute to impairment of epithelial barrier function in patients with GERD (Kandulski et al., 2010; Shan et al., 2012; Yoshida et al., 2007). Although it seems that trypsin also plays important roles in esophageal function, it remains to be determined how trypsin affects esophageal motility.

The present study thus aimed to investigate the effects of trypsin on the esophageal motility, by focusing on how trypsin affects the contractility of the lower esophageal sphincter (LES), using preparations of LES circular smooth muscle isolated from porcine esophagus and endeavored to elucidate the underlying mechanisms since LES plays a key role in coordinated esophageal motility. We demonstrate, for the first time, that trypsin causes a unique biphasic response in the porcine LES, composed of an initial transient contraction and a subsequent relaxation. The present study also demonstrates a major contribution of PAR_2 to the trypsin-induced biphasic regulation and provides evidence for the mechanisms underlying these effects.

2. Materials and methods

2.1. Tissue preparation

The section of the porcine esophagus containing the esophagogastric junction was freshly obtained from a local slaughterhouse and immediately transported to our laboratory in normal extracellular solution (NES). The specimen was cut open in the longitudinal direction along the greater curvature of the stomach and pinned, mucosal side up, to the flat surface of a silicone rubber plate. After removing the mucosal layer, the circular smooth muscle sheets of the LES were excised and cut into strips (2 mm × 5 mm) under a binocular microscope.

2.2. Measurement of force produced by LES circular smooth muscle strips

The LES circular smooth muscle strips were mounted vertically to a TB-612T force transducer (Nihon Koden, Tokyo, Japan) in a 5 ml organ bath containing NES, and the measurement of isometric force in the strips was performed at 37 °C. During the equilibration period, strips were first stretched to 1.3-fold resting length and were then stimulated with 118 mM K^+ extracellular solution (118-KES) every 10 min. The resting level of force spontaneously and gradually increased (2.185 ± 0.392 mN) during the equilibration period and then declined to a steady level (0.921 ± 0.255 mN). During the equilibration period, the contractile response to 118-KES also gradually increased with repeated stimulation, while a steady response was obtained within 2 h. All experimental protocols were then initiated. The extent of force development is expressed in mN, with zero being assigned to the resting level of force just prior to the initiation of the experimental protocols.

2.3. Immunohistochemistry of PAR_2 in LES circular smooth muscle

A segment of the porcine esophagus containing the LES was fixed and embedded in paraffin. Paraffin-embedded sections, 4 μm thick, were prepared. Sections were deparaffinized in xylene, and rehydrated in ascending dilutions of ethanol. The samples were incubated overnight at 4 °C with anti-PAR_2 antibody (1:50 dilution) in PBS containing 3% non-fat dried milk. For a negative control, the overnight incubation was performed without primary antibody. After 40-min incubation at room temperature with a horseradish peroxidase-conjugated secondary antibody, the sections were developed with 3-3′-diaminobenzidine, and counter-stained with hematoxylin.

2.4. Solutions and drugs

The composition of NES was (in mM): 137.4 NaCl, 5.9 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 11.5 glucose, and 11.6 HEPES, pH 7.3. KES was prepared by replacing NaCl with equimolar KCl. All chemicals were reagent grade unless otherwise indicated. Trypsin, thrombin, tetrodotoxin, GF109203x, chelerythrine, apamin, iberiotoxin, glibenclamide, 4-aminopyridine (4-AP) and p-amidinophenylmethanesulfonyl fluoride (p-APMSF) were obtained from Sigma (St. Louis, MO, USA). TFLLR-NH_2 (PAR_1-AP; PAR_1-activating peptide), SLIGRL-NH_2 (mouse PAR_2-AP), SLIGKV-NH_2 (human PAR_2-AP), AYPGKF-NH_2 (PAR_4-AP), and FSLLRY-NH_2 (PAR_2 antagonist) were purchased from Bachem (Bubendorf, Switzerland). AC-264613 was purchased from Tocris (Bristol, UK). ENMD-1068 was purchased from Abcam (Cambridge, UK). Y-27632 and SB203580 were purchased from Calbiochem (San Diego, CA, USA). PD98059 and H-1152 were purchased from Cayman Chemical (Boston, MA, USA). SCH79797 and anti-PAR_2 monoclonal antibody (SAM11) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). N^{ω}-nitro-L-arginine methyl ester (L-NAME) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.5. Data analysis

All data are expressed as the mean ± S.E.M. Student's *t*-test was used to determine statistical significance between two groups. An analysis of variance followed by Dunnett's test was used to determine statistical significance between more than two groups. A value of $P < 0.05$ was considered to be significant.

3. Results

3.1. Effects of trypsin on the tone of porcine LES circular smooth muscle

Trypsin, at concentrations up to 100 nM, had no effects on the tone of the LES circular smooth muscle (Fig. 1C). At 300 nM, trypsin induced a transient contraction. At concentrations of 1 μM and higher, the relaxant effect became apparent and trypsin induced a biphasic response consisting of a transient contraction and a subsequent transient relaxation (Fig. 1A and C). An apparent maximal contractile response was observed at 1 μM trypsin (0.747 ± 0.088 mN, $n=16$). At this concentration, the force peaked 3.17 ± 0.41 min ($n=16$) after initiating trypsin stimulation (Fig. 1A). At concentrations higher than 1 μM, the level of contraction obtained with 3 μM and 10 μM was smaller than that obtained with 1 μM (Fig. 1C). However, the time to the peak contraction (1.88 ± 0.22 min, $n=11$) seen with 10 μM trypsin was significantly shorter than that obtained with 1 μM (Fig. 1A and B). On the other hand, trypsin-induced relaxation increased in a concentration-dependent manner at concentrations of 1 μM and higher. An apparent maximal relaxant effect was obtained with 10 μM trypsin (−0.699 ± 0.106 mN, $n=15$). With 10 μM trypsin, the level of force reached a minimal level during the relaxation phase, 9.97 ± 0.84 min ($n=11$) after initiating stimulation (Fig. 1B).

Pretreatment with 1 μM tetrodotoxin had no effects on the spontaneous change in resting force, or on the trypsin-induced contraction and relaxation responses (data not shown). Pretreatment with carbenoxolone, an inhibitor of gap junction, also had no effects on trypsin-induced contractile and relaxant responses