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

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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, 118mM K^+ extracellular solution; p-APMSF, p-amidinophenyl methanesulfonyl fluoride; AP, activating peptide; L-NAME, N^{ω}-nitro-L-arginine methyl ester.

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.

Keywords: Esophageal motility; Trypsin; Proteinase-activated receptors; Gastro-esophageal Reflux Disease**;** Smooth muscle

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.

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 a 40-min incubation at room temperature with a horseradish peroxidase-conjugated secondary antibody, the sections were developed with 3-3′-diaminobenzidine, and counterstained 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). Y27632 and SB203580 were purchased from Calbiochem (San Diego, CA, USA). PD98059 and H1152 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 (data not shown). These observations exclude major contributions of the enteric nervous system and the interstitial cells of Cajal on the contractile and relaxant effects of trypsin in the LES circular smooth muscle. Furthermore, trypsin, at concentrations up to 10 μM, did not affect the following contractile

responses to 118-KES depolarization or carbachol, thus ruling out the contribution of cytotoxic effect to the trypsin-induced contractile and relaxant responses.

3.2. Involvement of PAR_2 in the trypsin-induced response of porcine LES circular smooth muscle

Proteinase activity is a prerequisite for the involvement of PARs in proteinase-induced cellular effects. When trypsin was pretreated with 100 μM p-APMSF, a serine protease inhibitor, both contractile and relaxant effects of trypsin were abolished (Fig. 2A). We next examined which PARs are involved in trypsin-induced responses. PAR_1-AP (TFLLR-NH_2) induced a substantial contraction (3.018 ± 0.735 mN, n=5) at a concentration of 100 μM. However, pretreatment with 10 μM SCH79797, a PAR_1 antagonist, had no inhibitory effects on this contraction (2.822 ± 1.235 mN, n=4) (Fig. 2D). Thrombin, an endogenous PAR_1 agonist, had no contractile or relaxant effects, up to 10 U/ml (data not shown). Moreover, pretreatment with 10 μM SCH79797, a PAR_1 antagonist, had no inhibitory effects on trypsin (10 μM)-induced contraction and relaxation (Fig. 2E). Therefore, the involvement of PAR_1 in the trypsin-induced responses is unlikely. Second, two PAR_2-APs, SLIGRL-NH_2 and SLIGKV-NH_2, induced significant contraction. The levels of contraction obtained with 100 μM SLIGRL-NH_2 (0.225 ± 0.118 mN, n=4) and 100 μM SLIGKV-NH_2 (0.274 ± 0.118 mN, n=4) were similar to those obtained with 300 nM trypsin (Fig. 1C), but lower than those obtained with 1 μM trypsin (Fig. 2D). The level of contraction obtained with 1 mM SLIGKV-NH_2 (0.813 ± 0.267 mN, n=4) was equivalent to that (0.747 ± 0.088 mN, n=16) obtained with 1 μM trypsin (Fig. 2B and D). However, PAR_2-APs did not induce relaxation, even at 1 mM. Thus, we further investigated the effects of AC-264613 (a non-peptide PAR_2 agonist). Application of 1 μM AC-264613 induced significant contraction (0.147 ± 0.028 mN,

n=4) similar to that seen with SLIGRL-NH_2 and SLIGKV-NH_2. However, AC-264613 induced no apparent relaxation, as was the case with SLIGRL-NH_2 and SLIGKV-NH_2. In contrast, pretreatment with 10 μM FSLLRY-NH_2 (a peptide PAR_2 antagonist) partially but significantly inhibited not only contraction but also relaxation induced by 10 μM trypsin. However, pretreatment with 5 mM ENMD-1068 (a non-peptide PAR_2 antagonist) did not inhibit contraction or relaxation induced by 10 μM trypsin (Fig. 2C and E). Finally, PAR_4-AP (AYPGKF-NH_2) induced no response in LES circular smooth muscle (Fig. 2D). These results suggest that PAR_2 is involved in the trypsin-induced responses. Indeed, immunohistochemical analysis revealed expression of PAR_2 in LES circular smooth muscle and the mucosa in the region of the LES (Fig. 2F). These positive signals were abolished in the absence of the primary antibody (Fig. 2G).

3.3. Contribution of Rho kinase to trypsin-induced contraction of LES circular smooth muscle

The maximal contractile response with a modest relaxation was induced by 1 μM trypsin (Fig. 1C); therefore, the mechanisms of trypsin-induced contraction were investigated at this concentration. The application of 10 μM Y-27632, a Rho kinase inhibitor, induced a significant reduction of the resting force, which gradually reached a new steady state level (−0.686 ± 0.098 mN, n=7) within 10 min (Fig. 3A and G). Subsequent stimulation with 1 μM trypsin in the presence of 10 μM Y-27632 induced a net development of force (0.225 ± 0.069 mN, n=7), which was significantly smaller than the control (0.747 ± 0.088 mN, n=16) (Fig. 3A and H). Similar results were obtained with 1 μM H-1152, another inhibitor of Rho kinase (Fig. 3B, G and H). Y-27632 and H-1152 reduced the contractile effect of 1 μM trypsin by 69.7% (n=7) and 67.1% (n=6), respectively. On the other hand, PD98059 (a MEK1/2 inhibitor), SB203580 (a p38MAPK inhibitor), GF109203x (a PKC inhibitor) and

chelerythrine (a PKC inhibitor) had no significant effect on either the resting force or trypsin-induced contraction (Fig. 3C–H).

3.4. Involvement of membrane hyperpolarization in trypsin-induced relaxation of LES circular smooth muscle

The maximal relaxant effect was induced by 10 μM trypsin (Fig. 1C); therefore, the mechanism of relaxation was investigated at this concentration (Fig. 4). The application of 100 μM L-NAME significantly enhanced the contractile response to 118-KES by 30 % (data not shown); however, it had no effect on the resting level of force. Subsequent stimulation with 10 μM trypsin induced a biphasic response of initial contraction followed by relaxation (Fig. 4A). The extent of both contraction (0.666 ± 0.195 mN, n=5; data not shown) and relaxation (−0.902 ± 0.314 mN, n=5; Fig. 4C and D) did not significantly differ from those obtained in the absence of L-NAME. On the other hand, stimulation with 40-KES induced an initial phasic contraction, followed by a sustained contraction (0.745 ± 0.182 mN, n=5; Fig. 4). The addition of 10 μM trypsin during the 40-KES-induced sustained contraction caused a small transient contraction, but no relaxation (Fig. 4A). This results suggests the involvement of K^+ channels in the trypsin-induced relaxation.

The absence of the relaxant effect of trypsin during 40-KES stimulation could be due to the increased level of force caused by 40-KES. Therefore, the effects of several K^+ channel blockers on trypsin-induced relaxation were investigated. The application of 10 μM glibenclamide (an ATP-sensitive K^+ channel blocker), 1 mM 4-aminopyridine (a voltage-gated K^+ channel blocker), or 1 μM apamin (a small conductance Ca^{2+}-activated K^+ channel blocker) had no significant effect on the resting level of force. However, 100 nM iberiotoxin (a large conductance Ca^{2+}-activated K^+ channel blocker) significantly decreased

the resting level. Glibenclamide and 4-aminopyridine, but not apamin or iberiotoxin, significantly attenuated the trypsin-induced relaxation (Fig. 4D). The net decreases in force induced by 10 μM trypsin in the presence of glibenclamide (−0.157 ± 0.088 mN, n=5) or 4-aminopyridine (−0.127 ± 0.088 mN, n=4) were significantly smaller than that seen in the absence of K^+ channel blockers (Fig. 4D).

3.5. Effects of Rho kinase, ERK1/2 and p38MAPK inhibitors on trypsin-induced relaxation in LES circular smooth muscle

The effects of Rho kinase, MEK1 and p38MAPK inhibitors on the biphasic response to 10 μM trypsin were examined, mainly to elucidate the mechanism of trypsin-induced relaxation (Fig. 5 and 6). The application of 10 μM Y-27632 or 1 μM H-1152, but not 10 μM PD98059, 10 μM SB203580, 10 μM GF109203x or 10 μM chelerythrine, substantially decreased the resting level of force (Fig. 5). Subsequent stimulation with 10 μM trypsin induced a significant contraction in the presence of all kinase inhibitors (Fig. 5 and 6A). The extent of the net development of contraction obtained in the presence of kinase inhibitors was not significantly different from that obtained in their absence (Fig. 6A). These observations were in clear contrast to those shown in Fig. 3, where 10 μM Y-27632 and 1 μM H-1152 substantially suppressed the development of force induced by 1 μM trypsin.

In contrast, the trypsin-induced relaxation was inhibited by pretreatment with 10 μM Y-27632, 1 μM H-1152, 10 μM SB203580 or 10 μM PD98059 (Fig. 5 and 6B). The net decreases in force seen in the presence of Y-27632 (−0.196 ± 0.078 mN, n=7), H-1152 (−0.216 ± 0.069 mN, n=6), PD98059 (−0.206 ± 0.139 mN, n=7) or SB203580 (−0.078 ± 0.206 mN, n=5) were significantly smaller than those seen in their absence (Fig. 6B). The

pre-treatment with 10 μM GF109203x or 10 μM chelerythrine had no significant effect on the relaxation induced by 10 μM trypsin (Fig. 6B).

4. Discussion

The present study demonstrates, for the first time, that trypsin uniquely regulates the tone of the porcine LES. Trypsin caused a biphasic response consisting of an initial transient contraction and a subsequent relaxation. The relaxant effect appeared to require higher concentrations of trypsin compared with those required for the contractile effect. Therefore, the relaxant response was not evident at 1 μM and lower concentrations, while the biphasic response was apparent at concentrations higher than 1 μM. The observations with tetrodotoxin and carbenoxolone suggest a negligible contribution from the nervous system and the interstitial cells of Cajal to both contractile and relaxant effects of trypsin. Both contractile and relaxant effects of trypsin thus appear to be attributable to a myogenic response. A trypsin-induced biphasic response was also reported in the mouse gastric fundus (Cocks et al., 1999) and small intestine (Kawabata et al., 1999; Sekiguchi et al., 2006). In these studies, the biphasic responses consisted of an initial relaxation followed by a contraction and the relaxant effects were observed at lower concentrations than those required for the contractile effects. In one study (Liu et al., 2010), the contractile effect was partly dependent on the release of contractile neurotransmitters from capsaicin-sensitive nerves, while in another study (Huang, 2007), it was shown to be resistant to tetrodotoxin. The myogenic biphasic regulation with initial contraction followed by relaxation thus appears to be unique to the LES.

Both contractile and relaxant effects of trypsin were abolished by pretreatment with a protease inhibitor. This observation is consistent with the involvement of PARs in both effects. PAR_1, PAR_2, and PAR_4 are potential receptors for trypsin, while PAR_1 and PAR_4 are also activated by thrombin (Hollenberg et al., 1997). Thrombin had neither contractile nor relaxant effects. No contractile or relaxant effects were observed for PAR_4-AP. However, a PAR_1-AP,

TFLLR-NH_2, induced significant contraction, but this contraction was resistant to a PAR_1 antagonist, SCH79797. Therefore, the contractile response seen with PAR_1-AP may not be mediated by PAR_1, but it could be a non-canonical effect. A similar SCH79797-resistant and, therefore, non-canonical effect of TFLLR-NH_2 has been reported in rat myometrium (Aman et al., 2005). Moreover, a PAR_1 antagonist had no inhibitory effects on trypsin-induced responses. Therefore, any major involvement of PAR_1 or PAR_4 in the contractile and relaxant effects of trypsin is unlikely.

Accordingly, PAR_2 is the only receptor among the PARs, which is responsible for trypsin-induced responses. In fact, the expression of PAR_2 was detected by immunohistochemistry in LES circular smooth muscle (Fig. 2F). Both PAR_2-APs (peptide PAR_2 agonists) and AC-264613 (a non-peptide PAR_2 agonist) consistently induced a contractile response. However, no relaxant effects were observed with these non-protease agonists at concentrations up to 1 mM for PAR_2-APs and 1 μM for AC-264613. The effects of higher concentrations of non-protease PAR_2 agonists were not investigated because of a technical limitation. The extent of contraction and the contraction pattern induced by non-protease PAR_2 agonists were similar to those seen with 300 nM trypsin. At this concentration, trypsin induced no apparent relaxation (Fig. 1C). It is therefore possible that the potency of non-protease PAR_2 agonists was too weak to replicate both the contractile and relaxant effects of trypsin. However, the possibility that the relaxant effect of trypsin is not mediated by PAR_2 cannot be ruled out. In contrast, a PAR_2 antagonist, FSLLRY-NH_2, partially but significantly inhibited not only contraction but also relaxation induced by 10 μM trypsin. This observation suggests the involvement of PAR_2 in both the contractile and relaxant effects of trypsin. It has been reported that FSLLRY-NH_2 blocks PAR_2 activation induced by trypsin by interfering with the interaction of the tethered ligand to its docking site

on PAR_2, but not by inhibition of trypsin to cleave PAR_2 (Al-Ani et al., 2002). Therefore, the results obtained with FSLLRY-NH_2 might support the involvement of PAR_2 in both the contractile and relaxant effects of trypsin. However, the observations with a non-peptide antagonist, ENMD-1068, might cause some controversy for the role of PAR_2 in the trypsin-induced effects. There is a concern regarding the potency of ENMD-1068 because its IC_{50} is approximately 3 mM (Lohman et al., 2012), and 5 mM ENMD-1068 was used in the present study. Taken together, the observations obtained with agonists and antagonists appear to provide a suggestive indication but not fully convincing support for the involvement of PAR_2 in the trypsin-induced contraction and relaxation of porcine LES circular smooth muscle. Alternatively, a PAR_2-independent mechanism could be involved in this process. Specifically, trypsin cleavage might produce peptide agonists that indirectly mediate the contractile and/or relaxant effects of trypsin. This mechanism might be consistent with the fact that relatively high concentrations of trypsin were required to elicit contractile and relaxant effects in the present study. However, the requirement for high concentrations of trypsin might be attributable to the expression of serine protease inhibitors (serpins) in the porcine LES. In fact, some serpins are expressed in the esophagus (Strik et al., 2002).

The contractile effect of trypsin was maximal at 1 μM, with minimal accompanying relaxation (Fig. 1A). The mechanisms of trypsin-induced contraction were therefore investigated using this contraction. Two chemically different ROK inhibitors, Y27632 and H1152, similarly and substantially inhibited the contraction induced by 1 μM trypsin. ROK inhibitors also decreased the resting level of tension. However, inhibitors of PKC, MEK1 and p38MAPK exerted no significant effect on either the resting level of tension or the contraction induced by trypsin. These observations suggest that ROK plays an important role in the trypsin-induced contraction and in maintenance of the resting tone in the LES. It is also

possible that the inhibition of the contractile effect of trypsin was due to the suppression of the resting tension, but not due to inhibition of contractile mechanism activated by trypsin. The observations of the present study are therefore consistent with those of a previous study, regarding the involvement of ROK in the maintenance of spontaneous tone in the cat LES (Harnett et al., 2005). However, the observations of the present study are inconsistent with those of a previous study, which showed a major role of PKC, ERK1/2 and p38MAPK in the acetylcholine-induced contraction of the circular smooth muscle of the cat LES (Cao et al., 2003).

The mechanisms of trypsin-induced relaxation were examined at 10 μM, which induced maximal relaxation. NO plays a central role in the vagal nerve-mediated relaxation of the LES in various species (Paterson et al., 1992). The effect of L-NAME and 40 mM K^+ suggest that the trypsin-induced myogenic relaxation is mainly attributable to membrane hyperpolarization. Furthermore, the inhibitory effects of glibenclamide and 4-aminopyridine suggest a major contribution of ATP-sensitive K^+ channels and voltage-gated K^+ channels to the trypsin-induced relaxation in the porcine LES. PAR_2-mediated relaxation has been shown to be mainly dependent on apamin-sensitive, small conductance Ca^{2+}-activated K^+ channels in mouse gastric fundus (Cocks et al., 1999), guinea pig colon (Cocks et al., 1999) and rat colon (Mule et al., 2002). There was, however, variation in the type of K^+ channel that mediates the trypsin-induced relaxation depending on the species and the type of gastrointestinal tract. The trypsin-induced relaxation was greatly suppressed by pre-treatment with Y27632, H1152, PD98059, or SB203580, indicating that K^+ channels, ERK1/2, p38MAPK, and ROK contribute to trypsin-induced relaxation.

The regulation of LES tone by trypsin/PAR_2 signaling pathway in the present study may have clinical relevance in GERD. GERD is defined as a condition, in which reflux of

stomach contents causes clinical symptoms, including heartburn and regurgitation as well as complications such as Barrett's esophagus and reflux esophagitis (Vakil et al., 2006). Not only acid reflux but also non-acid reflux contributes to the development of GERD symptoms (Vakil et al., 2006). Reflux of duodenal juices with trypsin into the esophagus is one of the major mechanisms for non-acid reflux (Orel and Vidmar, 2007; Vaezi and Richter, 1996). It was shown that trypsin concentration in pure pancreatic juice was nearly 40 mM in normal healthy subjects (Fedail et al., 1979). Especially it was reported that up to 26 % of patients with partial gastrectomy developed GERD symptoms and that trypsin concentration in esophagus was increased up to 428 mM (Gotley et al., 1992). Therefore, it is quite possible that reflux of trypsin into esophagus induces biphasic regulation of LES tone via activation of PAR_2, as observed in the present study. Although trypsin/PAR_2 signaling pathway is suggested to play a role in pathogenesis and pathophysiology of GERD (Kandulski et al., 2010; Souza, 2010), the pathophysiological roles of trypsin/PAR_2 in the regulation of LES tone thus remain to be elucidated in GERD patients.

In conclusion, trypsin induces a unique regulation of tone mainly by activating PAR_2 in the circular smooth muscle of the porcine LES. Trypsin exerts a contractile effect at lower concentrations than those required for the relaxant response, thus causing a biphasic response consisting of initial contraction and subsequent relaxation at concentrations higher than 1 μM. ROK is suggested to contribute to both contractile and relaxant responses, while ERK1/2 and p38MAPK contribute to the relaxant effect. The trypsin-induced relaxation is mediated by ATP-sensitive K^+ channels and voltage-gated K^+ channels. A trypsin/PAR_2 signaling pathway would be a potential new target for the treatment of esophageal motility disorders including GERD patients.

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Author contributions:

Y.T., E.I. and K.H. designed this study and mainly wrote the manuscript. Y.T. carried out physiological experiments. S.T. and Y.O. carried out pathological experiments. M.H., K.N. and H.A. provided technical and material supports. R.T. supervised this study.

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Figure legends

Fig. 1. Contractile and relaxant responses to trypsin in the circular smooth muscle of the porcine LES.

A, B, Representative recordings of the contractile and relaxant responses induced by 1 μM trypsin (A; n=16) and 10 μM trypsin (B; n=15). C, Concentration-dependent responses to trypsin (10 nM – 10 μM) are summarized. The level of force is expressed as mN by assigning the level of force at rest in NES to be 0 mN. The data are shown as the mean ± S.E.M. *P<0.05, **P<0.01 vs. the resting level (0 mN); $^{\#}$P<0.05 vs. contractile force induced by 1 μM trypsin

Fig. 2. Involvement of PAR_2 in trypsin-induced responses in the circular smooth muscle of the porcine LES.

A-C, Representative recordings of the contractile and relaxant responses to 10 μM trypsin in the presence of p-APMSF (A), the contractile responses to human PAR_2-AP (SLIGKV-NH_2) (B), and the contractile and relaxant responses to 10 μM trypsin in the presence of 10 μM FSLLRY-NH_2, a PAR_2 antagonist (C). D, Summaries of the contractile responses to PAR-APs including PAR_1-AP (TFLLR-NH_2) (n=5), mouse PAR_2-AP (SLIGRL-NH_2) (n=4), human PAR_2-AP (SLIGKV-NH_2) (n=4), AC-264613 (n=4), and PAP_4-AP (AYPGKF-NH_2) (n=4). Data are the mean ± S.E.M. of the numbers of experiments indicated above. **P<0.01 vs. control. E, Summaries of the effects of PAR_1 antagonist (SCH79797) (n=4) or PAR_2 antagonist (FSLLRY-NH_2) (n=4) on the contractile and relaxant responses to 10 μM trypsin. The resting level of force seen in NES was assigned to be 0 mN. The data are the mean ± S.E.M. of the numbers of experiments indicated above. *P<0.05 vs. control. F, G,

Microscopy images of immunohistochemistry with (F) and without (G) primary antibody, showing PAR_2 expression in the porcine LES. Scale bar indicates 1000 μm.

Fig. 3. Effects of protein kinase inhibitors on the contractile response to 1 μM trypsin in the circular smooth muscle of the porcine LES.

A-F, Representative recordings of contractile response to 1 μM trypsin in the presence of 10 μM Y-27632 (A; n=7), 1 μM H-1152 (B; n=6), 10 μM PD98059 (C; n=5), 10 μM SB203580 (D; n=4), 10 μM GF109203x (E; n=5) and 10 μM chelerythrine (F; n=4). G, H, Summaries of the effects of kinase inhibitors on the contractile response to 1 μM trypsin, as evaluated by the level of force, while assigning the resting level of force seen just prior to the application of inhibitors to be 0 mN (G) and the net development of force induced by trypsin (H). In G, the lower and upper levels of each column indicate results obtained just prior to trypsin stimulation and at the maximal contraction or at 3 min after trypsin stimulation when no apparent contraction was observed, respectively. The data are the mean ± S.E.M. of the numbers of experiments indicated above. *P<0.05, **P<0.01 vs. control.

Fig. 4. Effects of L-NAME, 40 mM K^+ depolarization and K^+ channel blockers on trypsin-induced relaxation in the circular smooth muscle of the porcine LES.

A, B, Representative recordings of the responses induced by 10 μM trypsin in the presence of 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (A; n=5) and 40 mM K^+ (B; n=5). C, D, Summaries of the effects of L-NAME and 40 mM K^+ (C) and K^+ channel blockers (D), including glibenclamide (n=5), 4-aminopyridine (n=4), apamin (n=6) and iberiotoxin (n=4) on trypsin-induced relaxation, as evaluated by the net changes in force induced by trypsin (C,

D). The data are the mean ± S.E.M. of the indicated numbers of experiments. *P<0.05, **P<0.01 vs. control.

Fig 5. Effects of protein kinase inhibitors on the biphasic response induced by 10 μM trypsin in the circular smooth muscle of the porcine LES.

Representative recordings of contractile and relaxant responses induced by 10 μM trypsin in the presence of 10 μM Y-27632 (A), 1 μM H-1152 (B), 10 μM PD98059 (C), 10 μM SB203580 (D), 10 μM GF109203x (E) and 10 μM chelerythrine (F) are shown. The level of force obtained at rest in NES was assigned to be 0 mN.

Fig. 6. Summary of effects of protein kinase inhibitors on the biphasic response induced by 10 μM trypsin in the circular smooth muscle of the porcine LES.

The summaries of the contractile (A), and relaxant responses (B) induced by 10 μM trypsin in the presence of 10 μM Y-27632 (n=7), 1 μM H-1152 (n=6), 10 μM PD98059 (n=7), 10 μM SB203580 (n=5), 10 μM GF109203x (n=5) and 10 μM chelerythrine (n=5), as evaluated by the net changes in force induced by trypsin (A, B). The data are the mean ± S.E.M. of the numbers of experiments indicated above. *P<0.05 vs. control.

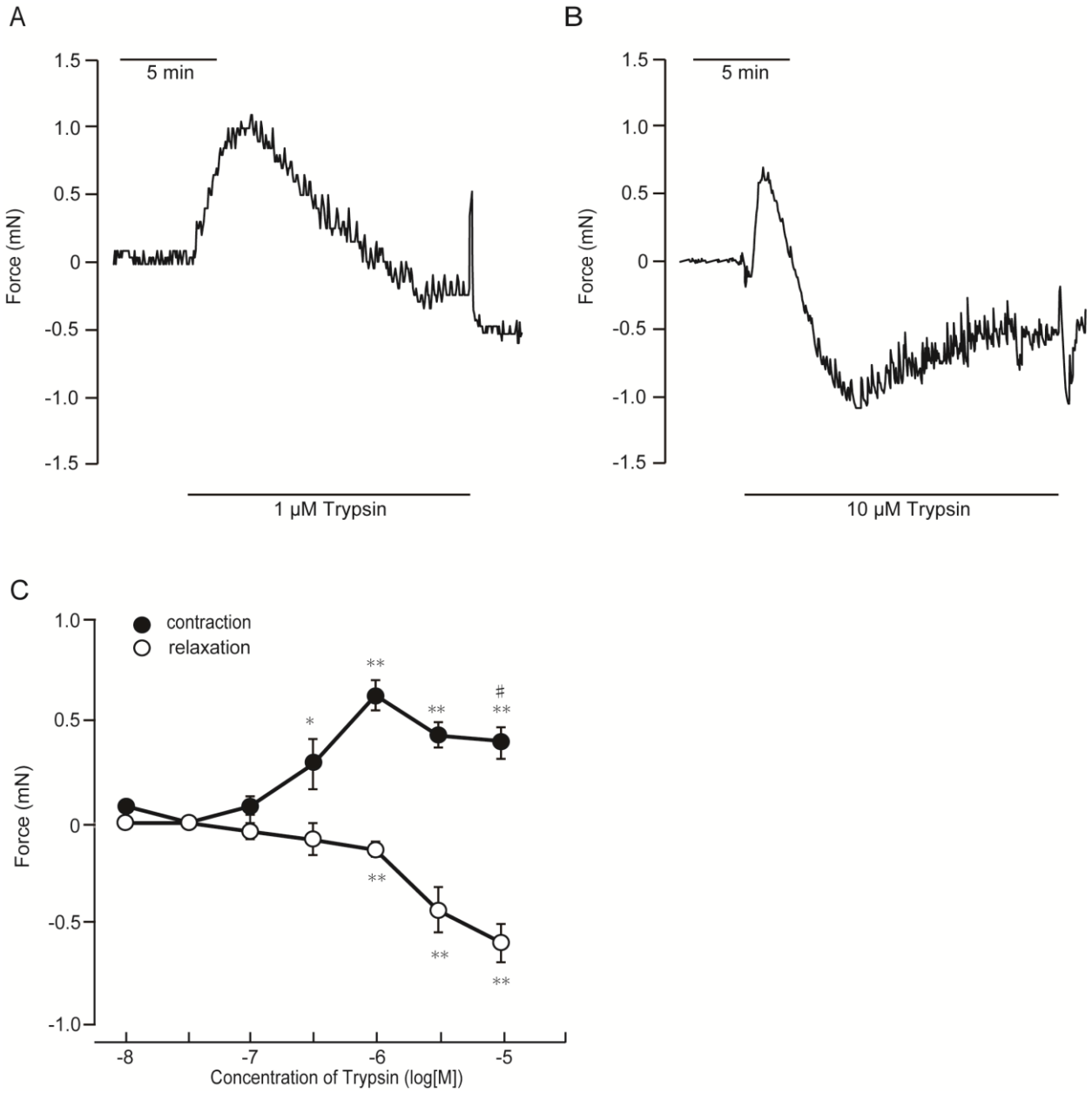
A
5 min
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
Force (mN)
1 μM Trypsin
B
5 min
Force (mN)
10 μM Trypsin
C
contraction
relaxation
Force (mN)
Concentration of Trypsin (log[M])
-8
-7
-6
-5

Figure1

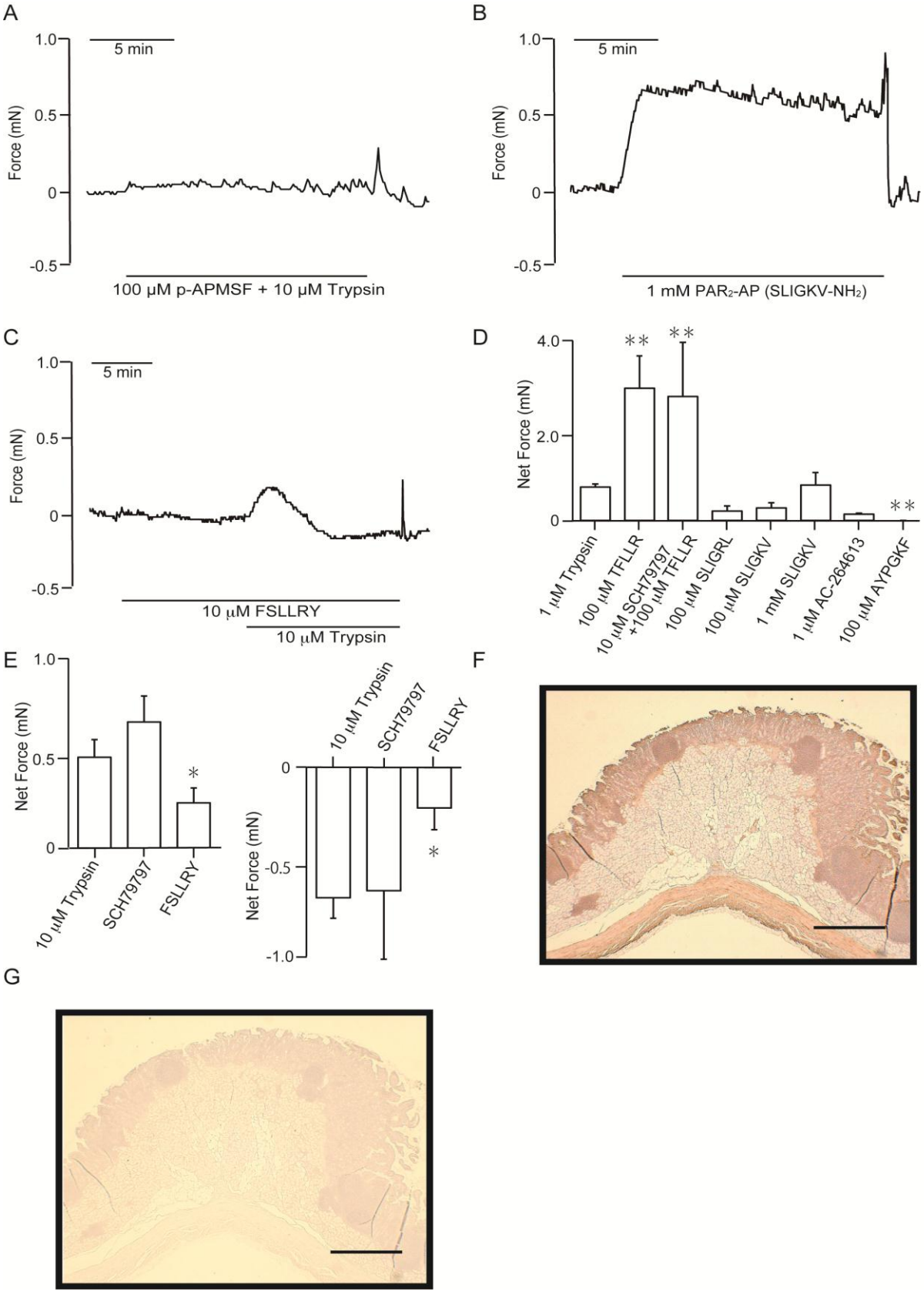
A
1.0
0.5
0
-0.5
Force (mN)
5 min
100 μM p-APMSF + 10 μM Trypsin
B
1.0
0.5
0
-0.5
Force (mN)
5 min
1 mM PAR2-AP (SLIGKV-NH2)
C
1.0
0.5
0
-0.5
Force (mN)
5 min
10 μM FSLLRY
10 μM Trypsin
D
4.0
2.0
0
Net Force (mN)
**
**
**
1 μM Trypsin
100 μM TFLLR
10 μM SCH79797 +100 μM TFLLR
100 μM SLIGRL
100 μM SLIGKV
1 mM SLIGKV
1 μM AC-264613
100 μM AYPGKF
E
1.0
0.5
0
Net Force (mN)
*
10 μM Trypsin
SCH79797
FSLLRY
0
-0.5
-1.0
Net Force (mN)
10 μM Trypsin
SCH79797
FSLLRY
*
F
G

Figure2

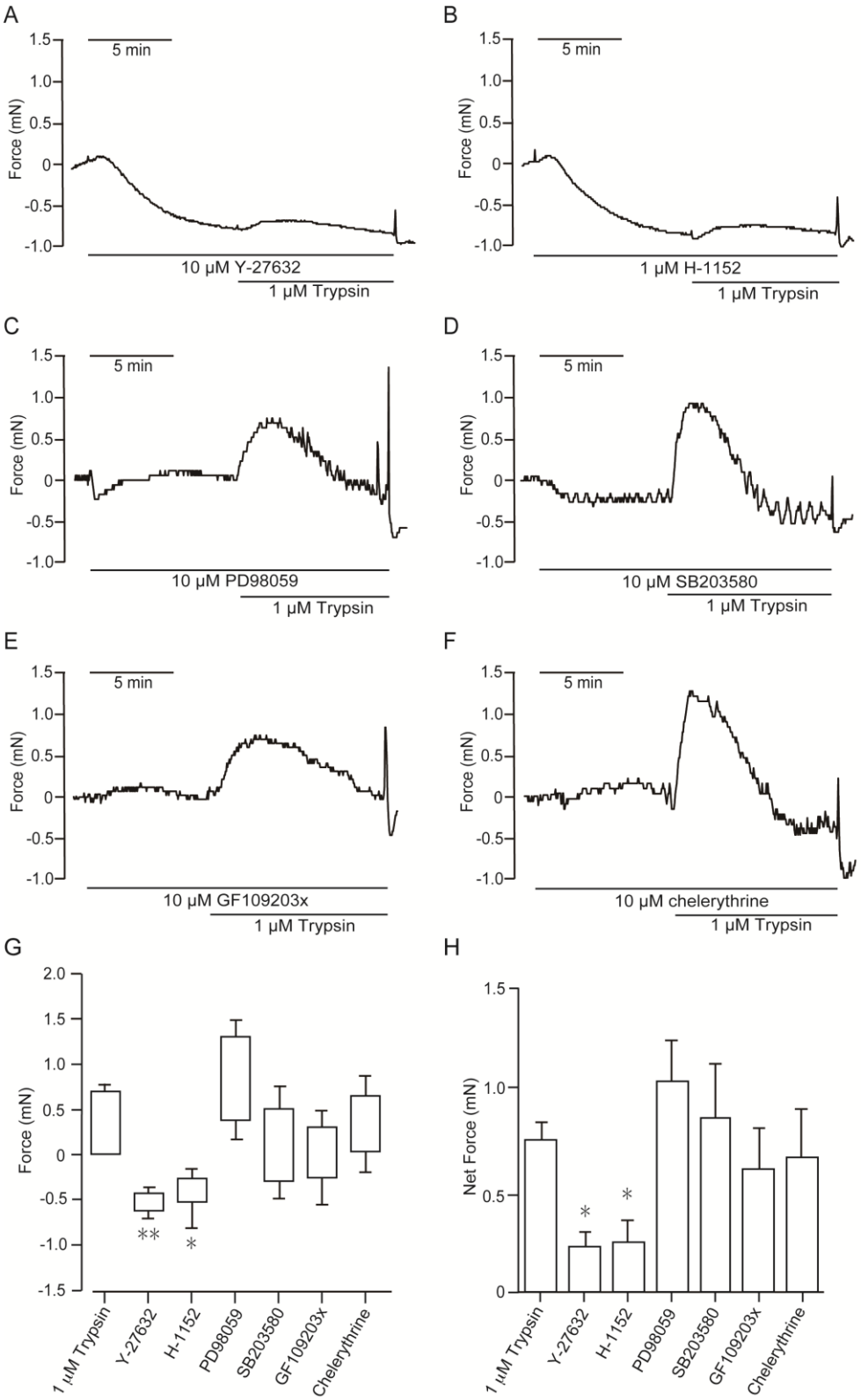
A
B
C
D
E
F
G
H
5 min
Force (mN)
1.5
1.0
0.5
0
-0.5
-1.0
10 μM Y-27632
1 μM Trypsin
1 μM H-1152
10 μM PD98059
10 μM SB203580
10 μM GF109203x
10 μM chelerythrine
Net Force (mN)
2.0
1.5
1.0
0.5
0
-0.5
-1.0
-1.5
1 μM Trypsin
Y-27632
H-1152
PD98059
SB203580
GF109203x
Chelerythrine

Figure3

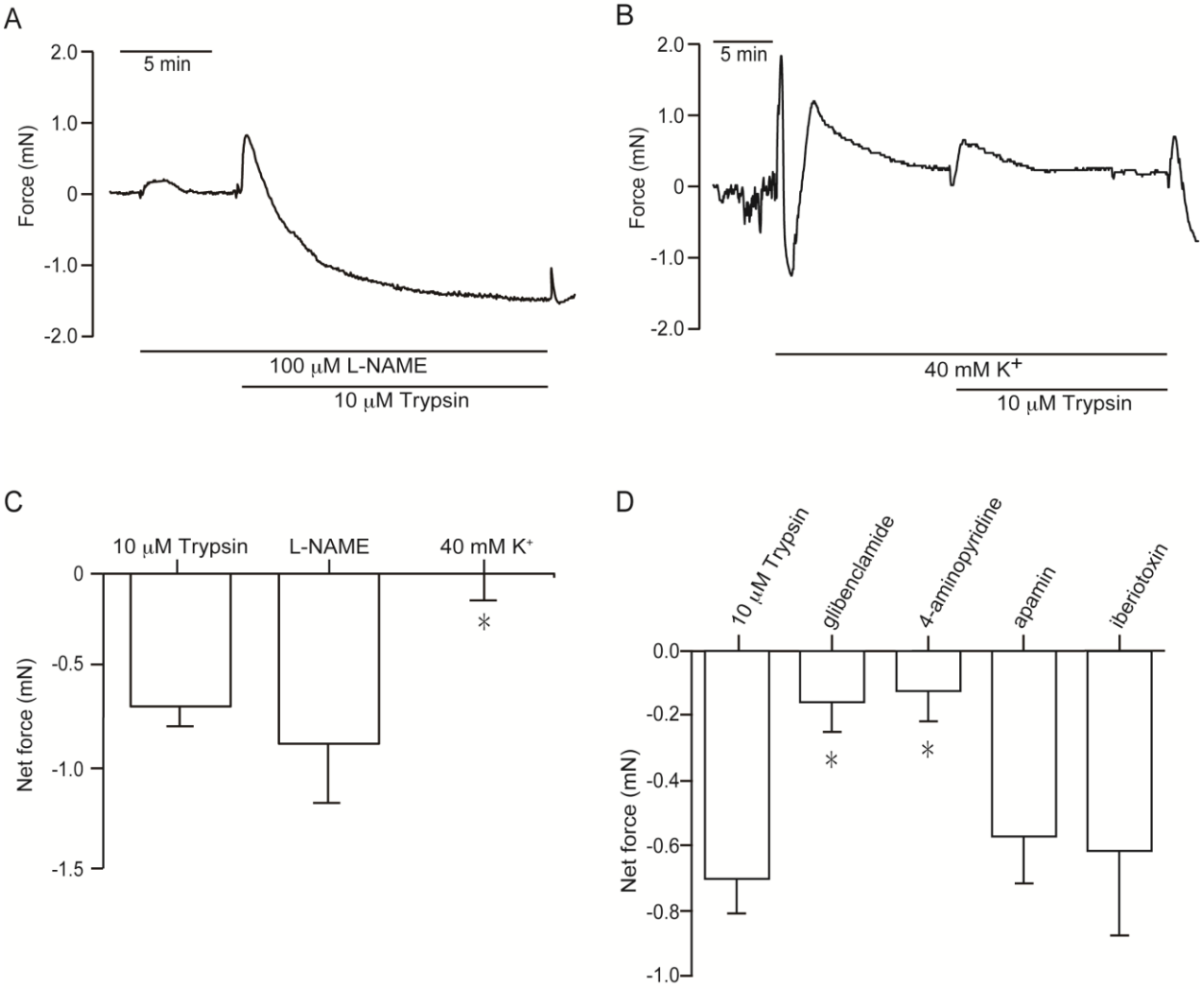
A
2.0
1.0
0
-1.0
-2.0
Force (mN)
5 min
100 μM L-NAME
10 μM Trypsin
B
2.0
1.0
0
-1.0
-2.0
Force (mN)
5 min
40 mM K+
10 μM Trypsin
C
10 μM Trypsin
L-NAME
40 mM K+
0
-0.5
-1.0
-1.5
Net force (mN)
*
D
10 μM Trypsin
glibenclamide
4-aminopyridine
apamin
iberiotoxin
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
Net force (mN)
*
*

Figure4

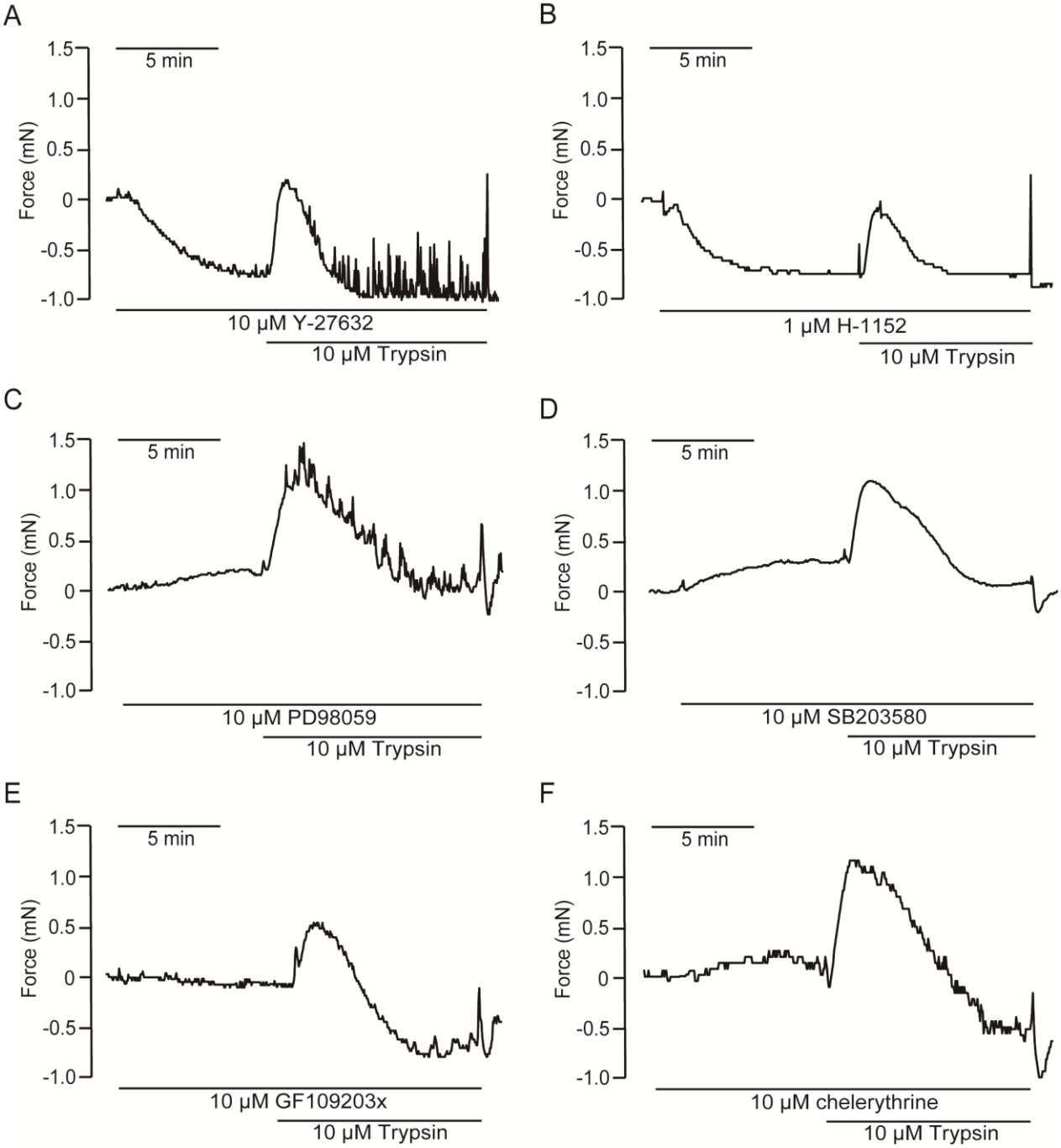
A
5 min
Force (mN)
1.5
1.0
0.5
0
-0.5
-1.0
10 μM Y-27632
10 μM Trypsin
B
5 min
Force (mN)
1 μM H-1152
10 μM Trypsin
C
5 min
Force (mN)
10 μM PD98059
10 μM Trypsin
D
5 min
Force (mN)
10 μM SB203580
10 μM Trypsin
E
5 min
Force (mN)
10 μM GF109203x
10 μM Trypsin
F
5 min
Force (mN)
10 μM chelerythrine
10 μM Trypsin

Figure5

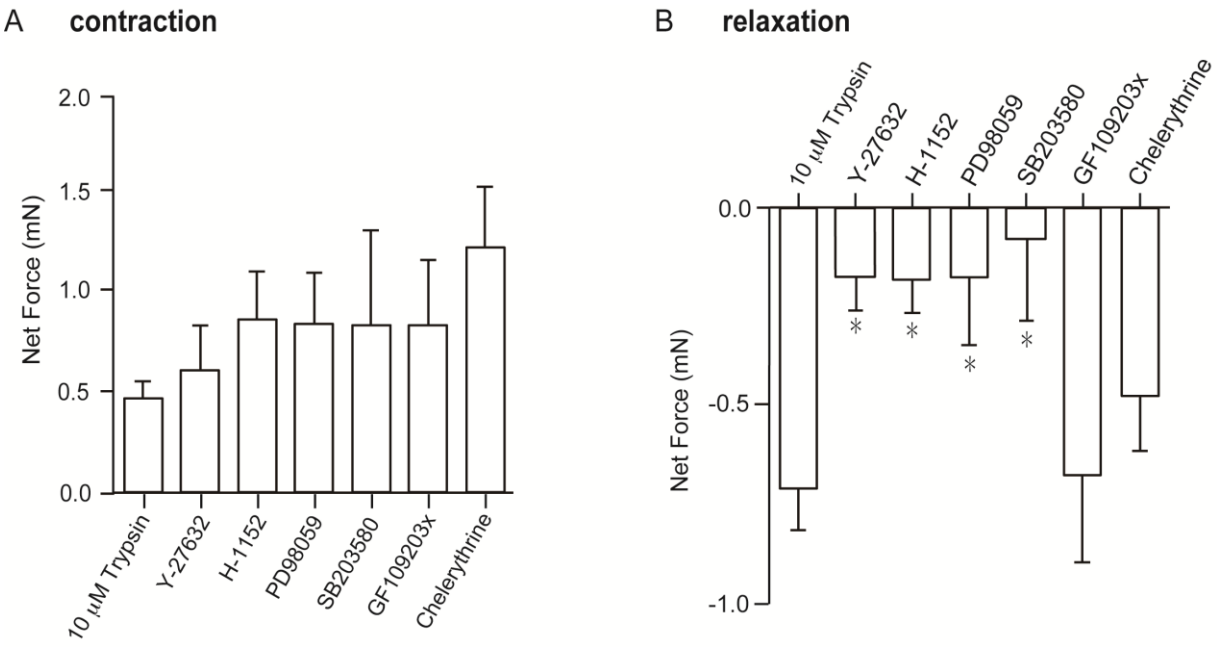
A
contraction
Net Force (mN)
2.0
1.5
1.0
0.5
0.0
10 μM Trypsin
Y-27632
H-1152
PD98059
SB203580
GF109203x
Chelerythrine
B
relaxation
Net Force (mN)
0.0
-0.5
-1.0
10 μM Trypsin
Y-27632
H-1152
PD98059
SB203580
GF109203x
Chelerythrine
*
*
*
*

Figure6