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Subepithelial trypsin induces enteric nerve-mediated anion secretion by activating proteinase-activated receptor 1 in the mouse cecum

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Abstract Serine proteases are versatile signaling molecules and often exert this function by activating the proteinase-activated receptors (PAR_1–PAR_4). Our previous study on the mouse cecum has shown that the PAR_1-activating peptide (AP) and PAR_2-AP both induced electrogenic anion secretion. This secretion mediated by PAR_1 probably occurred by activating the receptor on the submucosal secretomotor neurons, while PAR_2-mediated anion secretion probably occurred by activating the receptor on the epithelial cells. This present study was aimed at using trypsin to further elucidate the roles of serine proteases and PARs in regulating intestinal anion secretion. A mucosal–submucosal sheet of the mouse cecum was mounted in Ussing chambers, and the short-circuit current (I_{sc}) was measured. Trypsin added to the serosal side increased I_{sc} with an ED_{50} value of approximately 100 nM. This I_{sc} increase was suppressed by removing Cl^- from the bathing solution. The I_{sc} increase induced by 100 nM trypsin was substantially suppressed by tetrodotoxin, and partially inhibited by an NK_1 receptor antagonist, by a muscarinic Ach-receptor antagonist, and by 5-hydroxytryptamine-3 (5-HT_3) and 5-HT_4 receptor antagonists. The I_{sc} increase induced by trypsin was partially suppressed when the tissue had been pretreated with PAR_1-AP, but not by a pretreatment with PAR_2-AP. These results suggest that the serine protease, trypsin, induced anion secretion by activating the enteric secretomotor nerves. This response was initiated in part by activating PAR_1 on the enteric nerves. Serine proteases and PARs are likely to be responsible for the diarrhea occurring under intestinal inflammatory conditions.

Keywords Serine protease · Inflammation · Diarrhea · Eicosanoid · 5-Hydroxytryptamine · Substance P

Introduction

Intestinal fluid secretion is mainly derived from electrogenic anion secretion with its accompanying obligate of Na^+ and water. It is important to the physiology of digestion and absorption of nutrients and also for surface lubrication to enable the luminal contents to pass through smoothly. It is also believed to play a role in pathological conditions by flushing out noxious luminal agents [1]. Intestinal anion secretion is controlled by a variety of luminal and subepithelial substances (or by conditions such as mechanical distortion) that may have originated exogenously or be derived from the host itself. They may directly affect the epithelium, but their effects may also be mediated by various neurocrine, paracrine, and endocrine systems. The enteric nervous system, particularly the submucosal nerves, plays a vital role in the neurocrine regulation of intestinal anion secretion [1–3].

Proteases are not merely protein-degrading enzymes, but are now viewed as important signaling molecules that have fundamental roles in a wide variety of physiological processes, and are also associated with multiple disease conditions, including inflammation and cancer [4–9]. Nearly

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one-third of mammarian proteases are serine proteases, a class that includes trypsin, thrombin, kallikrein, and also type II transmembrane serine protease [8, 9]. Several previous studies have demonstrated in vitro in an Ussing chamber that trypsin and thrombin induced intestinal electrogenic anion secretion [10–14].

The signaling functions of serine proteases are often mediated by the proteinase-activated receptors (PAR_1–PAR_4). The PAR family members, particularly PAR_1 and PAR_2, are expressed throughout the gastrointestinal tract on epithelial cells, smooth muscle cells, and enteric nerves [15–17]. The role of PAR_1 and PAR_2 in regulating intestinal anion secretion has been demonstrated in several isolated intestinal mucosa by using the PAR_1-activating peptide (AP) and PAR_2-AP [10–13, 18, 19]. In addition, the activation of PAR_2 has been implicated in trypsin-induced anion secretion in the mouse distal colon, rat jejunum, and human colon [10, 12, 13]. However, the precise role of PARs in the serine protease-mediated regulation of intestinal transport had remained obscure and was thus investigated in the present study.

The cecum is the largest segment of the large intestine in rodents and many other mammals, and is highly active in luminal fermentation by bacteria as well as in epithelial transport [20, 21]. We have recently reported that both PAR_1- and PAR_2-APs added to the serosal side induced anion secretion in the mouse cecum in vitro in an Ussing chamber, whereby PAR_1-AP-mediated anion secretion occurred by activating submucosal secretomotor neurons, whereas PAR_2-AP-mediated anion secretion was by the direct activation of epithelial cells. The activation of anion secretion that was not mediated by the enteric nerve has been demonstrated in the same tissue by applying 10 μM trypsin to the serosal side [14]. Based on these findings, the present study was designed to further elucidate the mechanism for serosal trypsin to regulate intestinal anion secretion in the mouse cecum, and particularly the involvement of PAR_1, PAR_2, and the enteric nerves. The results suggest that, in contrast to 10 μM trypsin just mentioned, a moderate concentration of trypsin (100 nM) added to the serosal side induced anion secretion by stimulating the submucosal secretomotor nerve, this effect being at least partially due to the activation PAR_1, but not PAR_2. We also investigated the role of such neurocrine and paracrine mediators as Ach, 5-hydroxytryptamine (5-HT), substance P, and arachidonate metabolites in the 100 nM trypsin-induced anion secretion.

Materials and methods

Tissue preparation

All procedures used in this study were performed in accordance with the "Guiding Principles for Care and Use of Animals" approved by the Physiological Society of Japan and by the Institutional Animal Care Board at the University of Shizuoka. Male mice (30–40 g, Std:ddY; Japan SLC, Hamamatsu, Japan) were fed with standard food and water ad libitum until the time of the experiments. The animals were then killed by cervical dislocation and the cecum was excised. The resulting tissue was opened into a flat sheet, and the musculature was removed by blunt dissection to obtain the mucosa–submucosa preparation. The tissue was divided into four pieces of approximately equal size. One of these pieces was used in most experiments for determining the trypsin-induced response under control conditions, while the others were used for determining the trypsin-induced response under various treatment conditions.

Each piece was then mounted vertically between Ussing-type chambers that provided an exposed area of 0.2 cm^2. The volume of the bathing solution on each side was 5 ml, and the solution temperature was maintained at 37°C in a water-jacketed reservoir. The bathing solution contained (mM) NaCl, 119; $NaHCO_3$, 21; K_2HPO_4, 2.4; KH_2PO_4, 0.6; $CaCl_2$, 1.2; $MgCl_2$, 1.2; and glucose, 10 (pH 7.4). A Cl-free solution was provided by, respectively, using 119 mM Na-gluconate, 1.2 mM Mg-(gluconate)$_2$ and 8 mM Ca-(gluconate)$_2$ in place of 119 mM NaCl, 1.2 mM $MgCl_2$, and 1.2 mM $CaCl_2$. Each solution was bubbled with 95%O_2/5%CO_2.

Electrical measurements

The experiments were performed under short-circuit conditions. The short-circuit current (I_{sc}) and transmucosal conductance (G_t) were measured by using an automatic voltage-clamping device that compensated for the solution resistance between the potential-measuring electrodes (CEZ9100; Nihon Kohden, Tokyo, Japan) as previously described [22].

Chemicals

Bumetanide, procaine, 3-tropanyl-3,5-dichlorobenzoate, SB-204070 hydrochloride, atropine, hexamethonium, indomethacin, nordihydroguaiaretic acid (NDGA), thrombin from bovine plasma, trypsin from porcine pancreas, soybean trypsin inhibitor, and a P8340 protease inhibitor cocktail in DMSO were purchased from Sigma (St. Louis, MO, USA). Tetrodotoxin (TTX) was purchased from Calbiochem (La Jolla, CA, USA), and L-703,606 and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were purchased from Research Biochemical International (Natick, MA, USA). SKF-525A was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Mouse PAR_1 activating peptide (AP) SFFLRN-NH_2 and

mouse PAR_2 AP SLIGRL-NH_2 were purchased, respectively, from Yanaihara Institute (Fujimiya, Japan) and Bachem (Bubendaf, Switzerland). Indomethacin was dissolved in 21 mM $NaHCO_3$. Bumetanide, 3-tropanyl-3,5-dichlorobenzoate, NDGA, and SKF-525A were dissolved in dimethyl sulfoxide, before being administered to the bathing solution, the final concentration of dimethyl sulfoxide being 0.1%. The other chemicals were each applied from an aqueous stock solution.

Data and statistical analysis

Results are expressed as a percentage of the control response determined for each animal. Each value is presented as the mean ± SE, with n representing the number of animals. Statistical comparisons were made by Student's paired t test, significance being accepted at $P < 0.05$.

Results

Electrical responses to serosal trypsin

The mucosa–submucosa preparation of the mouse cecum in the Ussing chamber exhibited a basal short-circuit current (I_{sc}) value of 92 ± 6 μA cm^{-2} and a transmural conductance (G_t) value of 20.8 ± 0.4 mS cm^{-2} ($n = 78$) 20–30 min after starting the incubation. The I_{sc} value, but not G_t, decreased thereafter at the rate of approximately 10% per 10 min.

The addition of trypsin to the serosal solution caused changes in the I_{sc} and G_t values (Fig. 1a). I_{sc} was increased rapidly, reaching its peak about 5 min after adding a low concentration of trypsin (10–100 nM), and even more rapidly as the concentration was further increased. The peak increase in I_{sc} was followed by a decrease which, at concentrations of trypsin of more than 1 μM, reached the nearly basal level within 5–20 min. G_t increased essentially in parallel with I_{sc} after adding trypsin. It was noted, however, that 10–100 μM serosal trypsin evoked a late decrease in G_t to a level below the baseline. We have not explored this effect, but increases in the electrical resistance (=a decrease in G_t) and barrier property through the tight junction by extracellular serine protease have been reported in intestinal and other epithelial tissues [23–26]. In addition, 10–100 μM of serosal trypsin subsequently caused a marked and continuing increase in G_t in some, but not all preparations, which may reflect possible morphological disorganization by strong proteolysis. Figure 1b shows the relationships between the concentration of serosal trypsin and the resulting I_{sc} and G_t changes (only the initial peak increases). The ED_{50} value was 50–100 nM for both the

I_{sc} and G_t increases. We mainly explored in this study the response to 100 nM serosal trypsin.

Ionic basis for the serosal trypsin-induced I_{sc} increase

We first examined the ionic basis for the I_{sc} increase induced by trypsin (Fig. 2). The increase in I_{sc} induced by
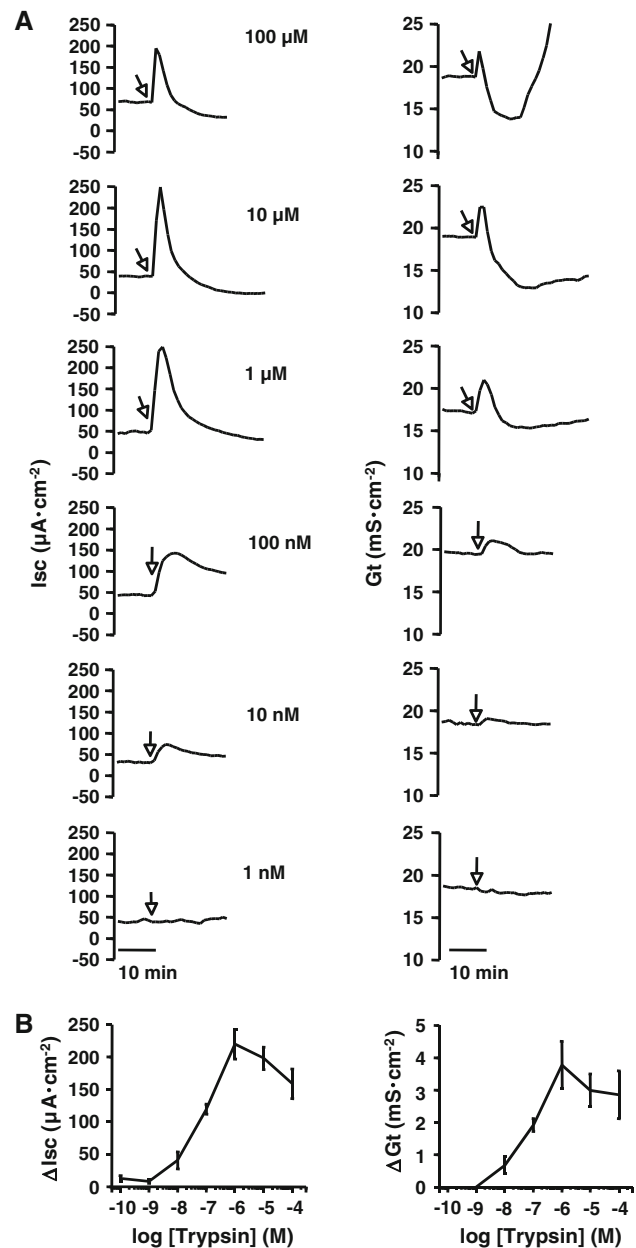

Fig. 1 Changes to I_{sc} and G_t induced by serosal trypsin. a Time-course characteristics for the changes in I_{sc} and G_t induced by 1 nM–100 μM trypsin added to the serosal side at the *arrowed* time ($n = 4$–8). Mean values are presented. b Concentration dependence of the serosal trypsin-induced increase in I_{sc} (ΔI_{sc}) and G_t (ΔG_t). Values were obtained when the peak I_{sc} increases were reached. Each data value is presented the mean ± SE, the number of animals used being 4–8

100 nM serosal trypsin was partially suppressed by 100 μM serosal bumetanide. In addition, Cl^- removal from both the mucosal and serosal bathing solutions almost abolished the trypsin-induced I_{sc} increase. Furthermore, mucosal NPPB, a Cl^- channel and/or transporter inhibitor (100 μM), largely suppressed the trypsin-induced I_{sc} increase. The trypsin-induced increase in G_t was substantially suppressed by Cl^- removal and NPPB (Fig. 3). Consequently, the increases in I_{sc} and G_t induced by 100 nM trypsin added to the serosal side were probably due to the activation of electrogenic anion secretion [1].

Involvement of the enteric nerves and other mediators

We next explored the role of the enteric nervous system in the anion secretion induced by trypsin. The I_{sc} and G_t increases induced by serosal 100 nM trypsin were substantially suppressed by pretreating the tissue with serosal TTX, indicating that they were mediated by the enteric submucosal nerves (Fig. 4; see also Fig. 3). The dependence on submucosal neurons of the I_{sc} and G_t increases induced by 100 nM serosal trypsin was supported by the finding that these increases were substantially suppressed by serosal but not by mucosal procaine, a local anesthetic (Fig. 4; see also Fig. 3).

We then elucidated the involvement of 5-HT by examining the effects of the $5\text{-}HT_3$-receptor antagonist, 3-tropanyl-3,5-dichlorobenzoate, and of the $5\text{-}HT_4$-receptor antagonist, SB-204070. The I_{sc} and G_t increases induced by 100 nM serosal trypsin was significantly

suppressed when the tissues had been pretreated with both the $5\text{-}HT_3$ and $5HT_4$ receptor antagonists (Fig. 4; see also Fig. 3).

Fig. 3 Changes in G_t (ΔG_t) induced by 100 nM serosal trypsin in the presence of various inhibitors. The values were obtained when the peak I_{sc} increases were reached. Each data value is presented as the mean ± SE, with $n = 3$–6. *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, and ***$P < 0.001$, compared with the control response that had been determined in the adjacent tissue by a paired t test
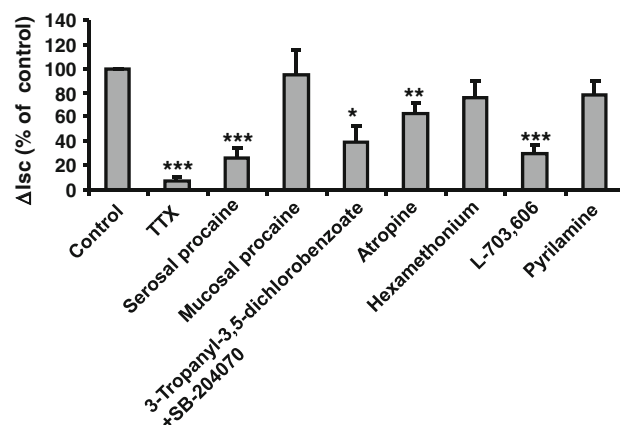
Fig. 4 Role of enteric submucosal neurons in serosal 100 nM trypsin-induced Cl^- secretion. Tetrodotoxin (*TTX*, 300 nM), 3-tropanyl-3,5-dichlorobenzoate (20 μM), *SB-204070* (10 μM), *atropine* (10 μM), *hexamethonium* (10 μM), *L-703,606* (10 μM) and *pyrilamine* (10 μM) were each added to the serosal side, and procaine (50 μM) was added either to the *mucosal* or *serosal* side 20 min before adding trypsin (100 nM) to the serosal side. The peak values for the increases in I_{sc} (ΔI_{sc}) induced by trypsin were determined, and are expressed as a percentage of the control response obtained in the adjacent tissue. Each data value is presented as the mean ± SE, with $n = 3$ for 3-tropanyl-3,5-dichlorobenzoate + SB-204070 (10 μM), $n = 6$ for mucosal procaine and pyrilamine, and $n = 5$ for the other compounds. *$0.01 < P < 0.05$, **$0.001 < P < 0.01$, and ***$P < 0.001$, compared with the control response by a paired t test
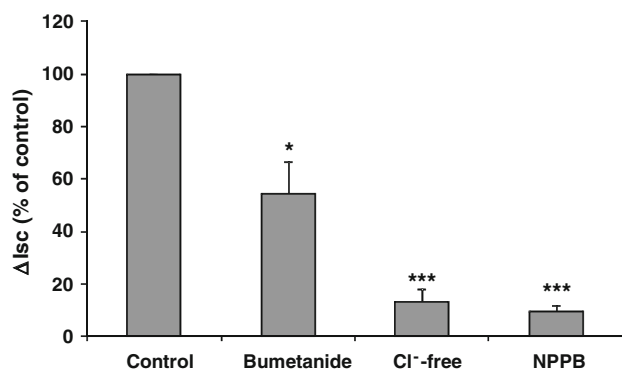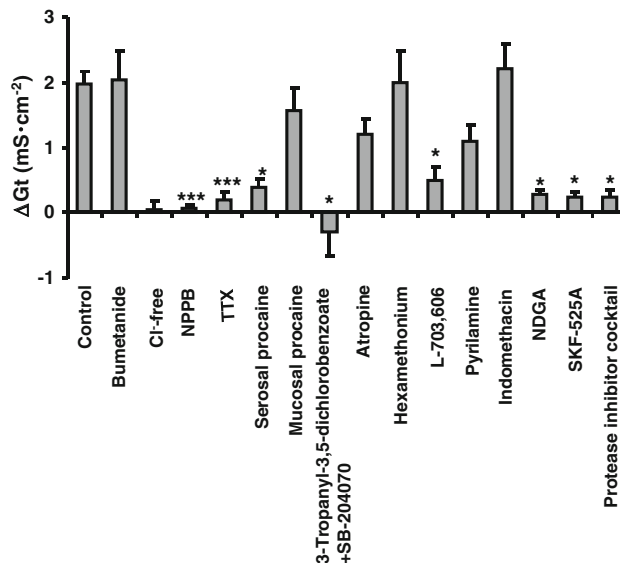

Fig. 2 Ionic basis for the increase in I_{sc} induced by 100 nM serosal trypsin. *Bumetanide* (100 μM, serosal) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (*NPPB*; 100 μM, mucosal) were added, or Cl^- was removed (from both the mucosal and serosal bathing solutions) 20 min before adding 100 nM trypsin to the serosal side. Peak values for the increases in I_{sc} (ΔI_{sc}) induced by trypsin were determined and are expressed as a percentage of the control response obtained in the adjacent tissue. Each data value is presented as the mean ± SE, with $n = 4$ for bumetanide and $n = 5$ for the other compounds. *$0.01 < P < 0.05$ and ***$P < 0.001$, compared with the control response that had been determined in adjacent tissue by a paired t test

We then examined the role of neurotransmitters Ach and tachykinin in the 100 nM serosal trypsin-induced anion secretion by using antagonists for the musocarinic and nicotinic acetylcholine receptors (atropine and hexamethonium) and that for the tachykinin NK_1 receptor (L-703,606). Atropine significantly reduced the increase in I_{sc}, but not G_t, induced by 100 nM serosal trypsin, while hexamethonium had a minor influence on the response (Fig. 4; also see Fig. 3). In contrast, the trypsin-induced increases in I_{sc} and G_t were both considerably decreased by L-703,606 (Fig. 4; also see Fig. 3). These results suggest that trypsin stimulated the release of substance P, resulting in activation of the NK_1 receptor, and some Ach release to activate the muscarinic receptor.

The H_1 histamine receptor antagonist, pyrilamine, slightly but insignificantly suppressed the trypsin-induced increase in I_{sc} (Fig. 4, ; also see Fig. 3)

Involvement of eicosanoids

We next explored the role of arachidonate metabolites (collectively called eicosanoids) in the serosal 100 nM trypsin-induced anion secretion by examining the effects of pretreating the tissue with indomethacin, an inhibitor of cyclooxygenase, nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenases, and SKF-525A, an inhibitor of cytochrome P450 monooxygenases, on the trypsin-induced anion secretion (Fig. 5; also see Fig. 3). The I_{sc} increase induced by trypsin was not significantly affected by indomethacin (10 μM, both mucosal and serosal sides), but was significantly inhibited by NDGA (50 μM, serosal side) and SKF-525A (30 μM, serosal side). These results suggest that arachidonate metabolites of the lipoxygenase and cytochrome P450 monooxygenase pathways are involved in the trypsin-induced response.

Role of the proteinase-activated receptors

The I_{sc} increase induced by 100 nM serosal trypsin was substantially suppressed by the protease inhibitor cocktail (Fig. 6). Furthermore, the presence of a soybean trypsin inhibitor (0.59 mg/ml) on the serosal side almost completely abolished the increase in I_{sc} induced by 100 nM serosal trypsin (Fig. 7d). The stimulatory effect of 100 nM serosal trypsin on the electrical response therefore required the proteolytic activity of trypsin.

We then explored the mediation of PAR_1 and/or PAR_2 in the action of trypsin, since our previous study has reported that both the PAR_1-AP and PAR_2-AP, but not PAR_4-AP, stimulated anion secretion in the mouse cecum when added to the serosal side [14]. Both PAR_1 and PAR_2 are known to be activated by trypsin [16, 27]. To this end we determined the change in the trypsin-induced response
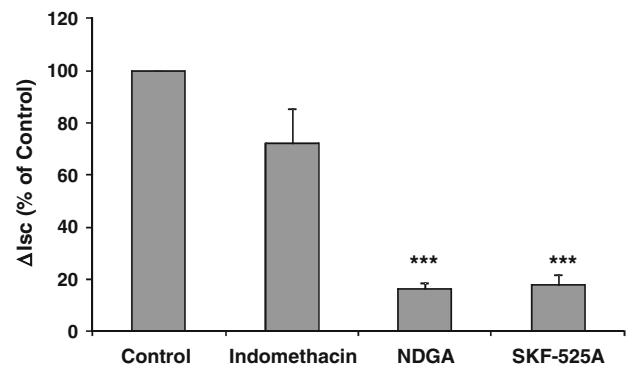

Fig. 5 Effect of inhibitors of arachidonic acid metabolism on I_{sc} induced by 100 nM serosal trypsin. *Indomethacin* (10 μM) was added to both the mucosal and serosal sides, and *NDGA* (50 μM) and *SKF-525A* (30 μM) were each added to the serosal side 20 min before adding trypsin (100 nM) to the serosal side. Each data value is presented as the mean ± SE, with $n = 5$. ***$P < 0.001$, compared with the control response that had been determined in the adjacent tissue by a paired t test
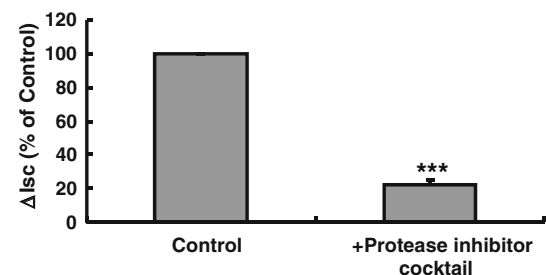

Fig. 6 Effect of the *protease inhibitor cocktail* (P8340; Sigma) on the I_{sc} increase induced by trypsin. The protease inhibitor cocktail was added to the serosal solution (diluted 1,000-fold) 20 min before adding 100 nM trypsin to the serosal solution. Peak values for the increases in I_{sc} (ΔI_{sc}) induced by trypsin were determined and are expressed as a percentage of the control response obtained in the adjacent tissue. Each data value is presented as the mean ± SE, with $n = 4$. ***$P < 0.001$, compared with the control response that had been determined in the adjacent tissue by a paired t test

after PAR_1 or PAR_2 desensitization. Figure 7a–c show that pretreating the tissue with 30 μM SFFLRN-NH$_2$ (a PAR_1-AP) to desensitize PAR_1 [14] significantly reduced the I_{sc} increase induced by serosal trypsin, suggesting that activation of PAR_1 on the enteric nerve is partially responsible for the trypsin-induced response. In addition, Fig. 7d–f show that the I_{sc} response induced by the PAR_1-AP was significantly decreased, but not abolished, when serosal trypsin (100 nM) had operated for 20 min before applying PAR_1-AP. Taking these results together, it is likely that serosal trypsin probably activated PAR_1 on the submucosal nerves and initiated the secretomotor reflex. On the other hand, pretreating the tissue with SLIGRL-NH$_2$, a PAR_2-AP, thereby desensitizing PAR_2 [14], did not suppress the I_{sc} increase induced by serosal trypsin, but rather
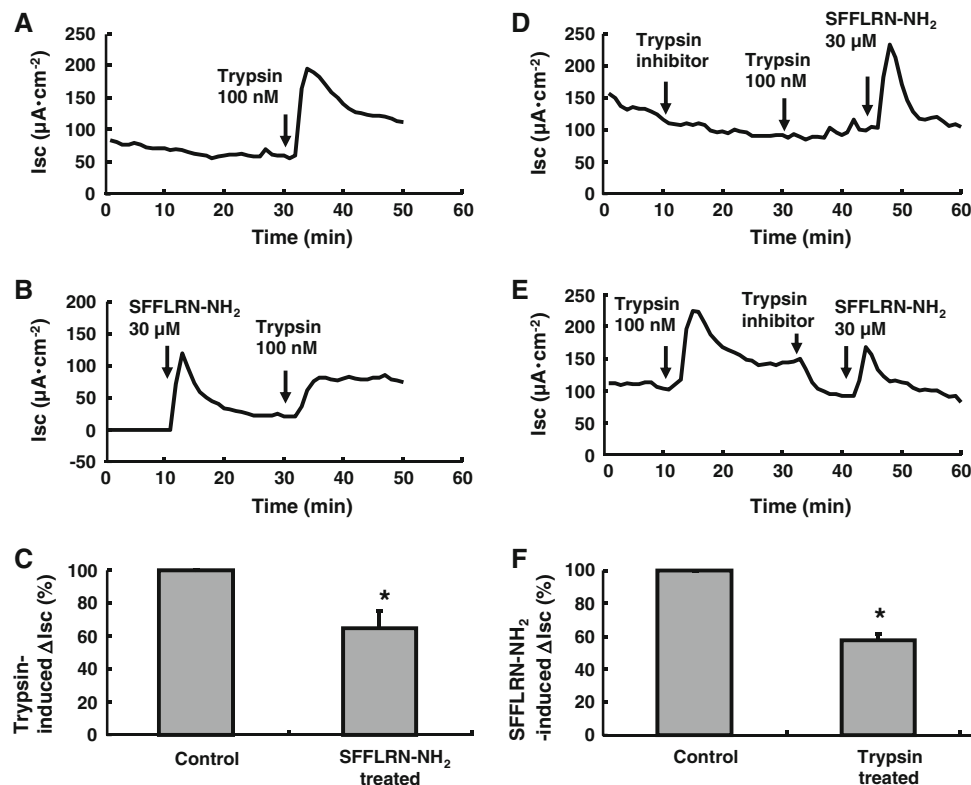
Fig. 7 Involvement of proteinase-activated receptor type 1 (PAR$_1$) in the serosal trypsin-induced I_{sc} increase. **a–c** Attenuation of the ΔI_{sc} induced by *trypsin* after pre-exposure to *SFFLRN-NH$_2$*, a PAR$_1$-activating peptide (AP). **a** The control ΔI_{sc} induced by serosal trypsin (100 nM). Mean values are presented, $n = 4$. **b** ΔI_{sc} induced by *trypsin* was obtained in the tissue that had been pretreated with serosal SFFLRN-NH$_2$. Mean values are presented, $n = 4$. **c** Peak values for ΔI_{sc} induced by serosal trypsin (100 nM) in the absence (control; shown in **a**) and presence (*SFFLRN-NH$_2$*; shown in **b**) of pre-exposure to PAR$_1$-AP. Each data value is presented as the mean $\pm$ SE. *$P < 0.05$, paired *t* test. **d–e** Attenuation of ΔI_{sc} induced by the serosal PAR$_1$-AP (30 μM), after pre-exposure to serosal

trypsin (100 nM). **d** The control ΔI_{sc} induced by SFFLRN-NH$_2$ was obtained in tissue that had been pretreated with trypsin, but only after the soybean trypsin inhibitor (0.59 mg/ml, serosal side) had been added. Mean values are presented, $n = 4$. **e** The soybean trypsin inhibitor was added after trypsin had worked for 20 min, and ΔI_{sc} induced by SFFLRN-NH$_2$ was finally obtained. Mean values are presented, $n = 4$. **f** The peak values for the increases in I_{sc} (ΔI_{sc}) induced by SFFLRN-NH$_2$ after pre-exposure to trypsin (*trypsin treated*; shown in **e**) are expressed as a percentage of the control response (control; shown in **d**) obtained in the adjacent tissue. Each data value is presented as the mean $\pm$ SE. *$P < 0.05$, paired *t* test

significantly enhanced it (Fig. 8). Although this enhancement is difficult to explain at the moment, PAR$_2$ activation may not have participated in the anion secretion induced by serosal 100 nM trypsin. The results thus suggest that other pathways not involving PAR$_1$ or PAR$_2$ were also involved in the trypsin-induced anion secretion.

Discussion

Effect of trypsin

The present results for the mouse cecum demonstrate that trypsin added to the serosal side induced electrogenic anion secretion with an ED$_{50}$ value of 50–100 nM. Previous studies on the mammalian small and large intestines in an

Ussing chamber have demonstrated that serosal trypsin could stimulate anion secretion with an ED$_{50}$ value of 10–100 nM in most cases, this being similar to the value obtained in the present study [10–14]. However, the mechanism for stimulating anion secretion by trypsin appears to be diverse according to the intestinal segment, the dose of trypsin, and probably animal species. For example, the response in the mouse distal colon and porcine ileum was abolished by TTX [11, 12], like the response to 100 nM trypsin in the mouse cecum that was observed in the present study. On the other hand, the anion secretion induced by serosal trypsin has not been inhibited by TTX in the human rectum [13]. In addition, our previous study on the mouse cecum has shown that, in sharp contrast to the moderate concentration used in the present study (100 nM), the activation of anion secretion induced
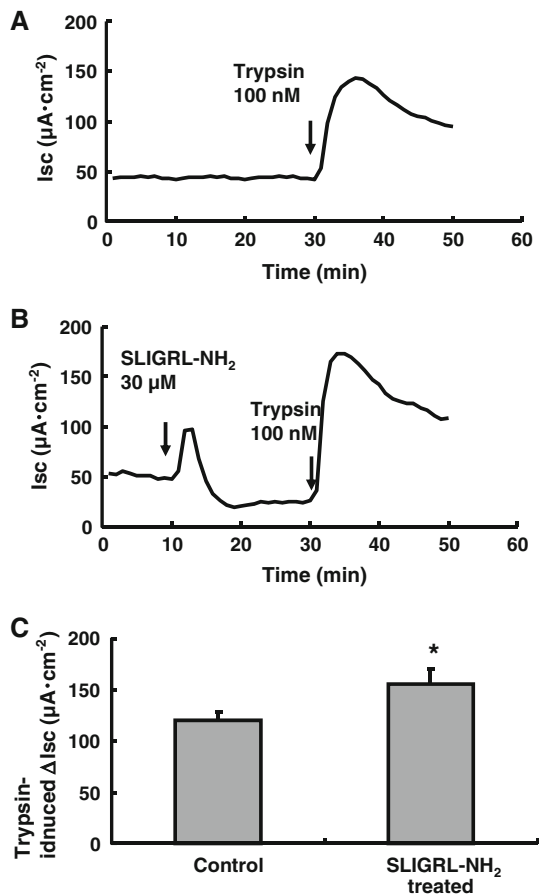
Fig. 8 Effects of pretreating by *SLIGRL-NH$_2$*, a PAR$_2$-AP, on the *trypsin*-induced increase in I_{sc} (ΔI_{sc}). **a** The control ΔI_{sc} value induced by serosal trypsin (100 nM). Mean values are presented, $n = 14$. **b** ΔI_{sc} induced by trypsin was obtained for tissue that had been treated with serosal SLGRL-NH$_2$. Mean values are presented, $n = 7$. **c** ΔI_{sc} values induced by serosal trypsin are presented in the tissue that had been pre-exposed to SLGRL-NH$_2$ or not. Each data value is presented as the mean ± SE. *$P < 0.05$, unpaired t test

by a high concentration of trypsin (10 μM) was not inhibited by TTX, indicating a dose-dependent difference in the action of trypsin [14]. Trypsin would be likely to induce the inhibition of Na and/or Cl absorption, in addition to the stimulation of anion secretion, since the stimulants of anion secretion in the intestines often simultaneously inhibit NaCl absorption to facilitate a net fluid secretion [1]. However, this likelihood has not yet been addressed.

Role of PARs

PAR$_1$ and PAR$_2$ have previously been demonstrated morphologically and functionally to be expressed in epithelial cells and/or enteric nerves and have been suggested to play a role in regulating intestinal secretion [10–14, 18, 19, 28, 29]. The results of the present study show that the I_{sc}

increase induced by trypsin was partially suppressed by pretreating with 30 μM PAR$_1$-AP, which had been shown to almost completely desensitize PAR$_1$ [14]. It is therefore likely that trypsin induced anion secretion at least in part by activating PAR$_1$, probably on the submucosal enteric nerve. Indeed, PAR$_1$-AP has been shown to stimulate enteric nerve-mediated anion secretion [14], and also to be expressed in submucosal nerves [19]. It is intriguing that the role of PAR$_1$ in the enteric nerve appears to be complex, since PAR$_1$-AP has been reported to inhibit the anion secretion evoked by stimulating enteric nerves in the mouse proximal colon, a segment next to the cecum [18].

We have previously shown that PAR$_2$-AP stimulates anion secretion that is not inhibited by TTX. Here, 100 nM serosal trypsin evoked almost no anion secretion when the tissue was treated with TTX (Fig. 4), thus excluding a major role for PAR$_2$ in trypsin-induced anion secretion. In agreement with this notion, desensitizing PAR$_2$ failed to reduce the subsequent response to trypsin (Fig. 8). Other pathways not involving PAR$_1$ or PAR$_2$ would therefore also be involved in the trypsin-induced anion secretion. The failure of 100 nM trypsin to activate PAR$_2$ and induce anion secretion in the mouse cecum was an unexpected finding, since PAR$_2$ was expressed in this preparation and PAR$_2$-AP can evoke anion secretion that is not mediated by enteric nerves [14]. In fact, a high concentration of trypsin (10 μM) can induce TTX-insensitive anion secretion [14]. Possibly, it was difficult, and therefore required a high concentration for large trypsin molecules to gain access to PAR$_2$ residing deep in the tissue, probably on epithelial cells. The involvement of PAR$_2$ in trypsin-induced anion secretion has been shown in the mouse distal colon and rat jejunum [10, 12, 13]. Taking this information together, PARs are likely to be present on various cell types and to play important roles in trypsin or other serine proteases regulating intestinal ion transport, but their precise roles are probably diverse according to the intestinal segment and animal species.

Neurocrine and paracrine mediators

The anion secretion induced by 100 nM serosal trypsin was partially inhibited by the muscarinic receptor antagonist, atropine, suggesting the involvement of Ach release from submucosal cholinergic secretomotor nerves [1–3, 30, 31]. The present results also suggest that activation of the NK$_1$ receptor by substance P, which was probably released from afferent nerves, would also be responsible in part for the anion secretion reflex induced by trypsin [14, 32–37]. NK$_1$ receptors have recently been shown to be abundantly expressed in subepithelial fibroblasts, although the relevance of this intriguing finding to the anion secretion induced by trypsin needs to be more fully investigated [38].

The anion secretion stimulated by 100 nM serosal trypsin was partially inhibited in the presence of both the 5-HT_3 receptor and 5-HT_4 receptor antagonists, suggesting the involvement of 5-HT release in this response [2, 3, 39–41]. 5-HT is mainly present in the epithelial enterochromaffin cells, but is also contained in the enteric nerve and mucosal mast cells [3, 39–41]. The mechanism by which trypsin stimulates any of these cells to release 5-HT remains for future study.

Arachidonic acid released from membrane phospholipids can be metabolized to generate various eicosanoids via three enzymatic routes: the cyclooxygenase, lipoxygenase and cytochrome P-450 epoxygenase pathways [42, 43]. The trypsin-induced anion secretion in the present study was substantially suppressed by NDGA (a lipoxygenase inhibitor) and SKF-525A (a cytochrome P-450 inhibitor), but not significantly affected by indomethacin (a cyclooxygenase inhibitor). This suggests that metabolites of the lipoxygenase pathway, hydroxyeicosatetraenoic acids (HETEs) and leukotrienes, and those of epoxygenase pathway, HETEs and epoxyeicosatrienoic acids (EETs), were involved in the response, but not that of the metabolites on the cyclooxygenase pathway, prostanoids. The intestinal mucosa has been shown to produce lipoxygenase metabolites [43, 44], which have been found to activate or inhibit anion secretion [45–50]. P450 expoxygenases and the production of HETEs and EETs have also been demonstrated in the intestinal tissues, although their role in regulating anion secretion is not known [42, 43, 51–53]. The cell types involved in releasing the lipoxygenase and epoxygenase metabolites of arachidonic acid in response to trypsin remain to be determined. Although prostanoids have hardly been involved in trypsin-induced anion secretion in the cecum, the anion secretion stimulated by PAR_1-AP or PAR_2-AP has been shown to be mediated via prostaglandin production in certain intestinal tissues [16, 43, 54–56].

Conclusion

The serine protease, trypsin, can induce anion secretion by activating the enteric secretomotor nerves in the mouse cecum. This response was initiated in part by activating PAR_1, probably on the enteric nerves. Other pathways not involving PAR_1 or PAR_2 would also have been involved, but their precise action remains for future investigation. Since serine proteases are known to be recruited and/or activated under certain pathological conditions, they are likely to play a role in the development of diarrhea that is often observed under such conditions [1, 57–63]. This reaction may be important for protecting the intestine, particularly the cecum, by washing harmful microbes and

their toxic products out of the lumen [21]. The natural proteases working in situ and the precise mode of their actions in regulating intestinal fluid secretion may vary under specific conditions and warrant further investigation.

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