Proteinase-activated receptors-1 and 2 induce electrogenic Cl⁻ secretion in the mouse cecum by distinct mechanisms

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Ikehara O, Hayashi H, Watanabe Y, Yamamoto H, Mochizuki T, Hoshino M, Suzuki Y. Proteinase-activated receptors-1 and -2 induce electrogenic Cl⁻ secretion in the mouse cecum by distinct mechanisms. Am J Physiol Gastrointest Liver Physiol 2010:G115–G125. First published April 22, 2010; doi:10.1152/ajpgi.00281.2009.—Proteinase-activated receptors (PARs) are cleaved by proteases. Previous in vitro studies on the mouse large intestine have indicated that PAR₁ and PAR₂ were involved in regulating epithelial ion transport, but that their roles were different between the proximal and distal colon. This present study was done to elucidate the roles of PAR₁ and PAR₂ in regulating anion secretion in the cecum, another segment of the large intestine. A mucosal-submucosal sheet of the mouse cecum was mounted in Ussing chambers, and the short-circuit current (Isc) was measured. The addition of a PAR₁-activating peptide (SFFLRN-NH₂) to the serosal surface increased Isc. This increase in Isc induced by SFFLRN-NH₂ was partially suppressed by serosal bumetanide and substantially suppressed by mucosal 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) and by the removal of Cl⁻ from the bathing solution. The Isc increase was also substantially suppressed by serosal tetrodotoxin (TTX) and neurokinin-1 receptor antagonist L-703,606 and was partially inhibited by serosal atropine and hexamethonium. The addition of a PAR₂-activating peptide (SLIGRL-NH₂) to the serosal surface also induced an increase in Isc; this increase was partially suppressed by bumetanide and substantially suppressed by NPPB and by the removal of Cl⁻, but not by TTX. The expression of mRNA for PAR₁ and PAR₂ was confirmed in the mucosa as determined by RT-PCR. In conclusion, PAR₁ and PAR₂ both induced Cl⁻ secretion in the mouse cecum. This secretion mediated by PAR₁ probably occurred by activation of the receptor on the submucosal secretomotor neurons, resulting mainly in the release of tachykinins and activation of the neurokinin-1 receptor, and partly in the release of ACh and activation of the muscarinic and nicotinic receptors. On the other hand, PAR₂-mediated Cl⁻ secretion probably occurred by activating the receptor on the epithelial cells. A variety of proteases would induce fluid secretion mediated by PAR₁ and PAR₂ in the cecum and thereby support bacterial fermentation and participate in mucosal inflammation.

Proteinases are known to function as signaling molecules. This function is, in many cases, mediated by proteinase-activated receptors (PARs) (25, 50). PARs belong to a family of G protein-coupled receptors that are activated by cleavage of their extracellular NH₂-terminal sequence by a variety of serine proteases. The newly exposed NH₂-terminal domain acts as a tethered ligand that binds residues in its own second extracellular domain to activate the cleaved receptor itself. The PAR family comprises at least four receptors, namely PAR₁ to PAR₄, which differ in respect to their protease specificity and tissue distribution (40, 47).

All members of the PAR family have been reported to be expressed in the gastrointestinal tract (19, 29, 35, 39, 40, 52). They are involved in almost all aspects of gastrointestinal physiology and pathophysiology, including motility, mucosal function, exocrine secretion, enteric nerve function, mucosal defense, and inflammation (5, 31, 40, 49, 50, 53). A role of PARs in regulating the epithelial ion transport function has been reported for PAR₁ and PAR₂. PAR₁ agonists stimulate Cl⁻ secretion in an intestinal cell line by activating the receptor on the cell (6, 7). On the other hand, in the mouse proximal colon, PAR₁ agonists probably inhibit neurally evoked Cl⁻ secretion by activating the receptor on the enteric nerve (8). The agonists for PAR₂ have been demonstrated to stimulate Cl⁻ secretion in intestinal cell lines (48). The stimulation of Cl⁻ secretion by PAR₂ agonists has also been shown in the rat jejunum, pig ileum, and human colon. Cl⁻ secretion in the rat jejunum and human colon is mediated by prostanoid release but not mediated by activation of the enteric nerve (38, 51), whereas Cl⁻ secretion in the pig ileum depends on activation of the submucosal nerve as well as on prostanoid release (23).

The purpose of this study was to elucidate the roles of PAR₁ and PAR₂ in regulating anion secretion in the mouse cecum. We particularly chose the cecum for the following three reasons: 1) the cecum is the active compartment, not merely for storage, because a large amount of short-chain fatty acids are produced in this segment by bacterial fermentation and absorbed (3, 33); 2) the cecum is exposed to a relatively large amount of proteases (activators of PARs) that have entered from the small intestine or have been derived from intraluminal bacteria (9, 41); and 3) the proximal and distal colons (of the mouse) have shown quite different epithelial responses to PAR₁ and PAR₂ stimulation (8, 18). We therefore mounted a mucosal-submucosal preparation of the cecum in Ussing cham-

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bers and measured the short-circuit current ($I_{sc}$) and transmucosal conductance ($G_t$). The results will show that both the PAR1-activating peptide (AP) and PAR2 AP evoked Cl$^-$ secretion. The PAR1 AP-induced secretion was mediated by enteric submucosal neurons, whereas PAR2 AP probably stimulated secretion by acting on epithelial cells.

**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* and were 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 (mucosal-submucosal sheet). The tissue was divided into four pieces of approximately equal size. One of them was used for determining the response under control conditions, whereas the others were used for the responses to various treatments. Each piece was 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 had the following composition (in mM): 119 NaCl, 21 NaHCO$_3$, 2.4 K$_2$HPO$_4$, 0.6 KH$_2$PO$_4$, 1.2 CaCl$_2$, 1.2 MgCl$_2$, and 10 glucose (pH 7.4). A Cl$^-$-free solution was provided by using 119 mM Na-gluconate, 1.2 mM MgCl$_2$, and 1.2 mM CaCl$_2$, respectively. 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 described previously (36).

**Conventional RT-PCR.** Total RNA was prepared either from the cecal mucosal-submucosal sheet or from isolated cecal epithelia. The mucosal-submucosal sheet was prepared in the same way as that for the Ussing chamber experiments. To isolate the epithelia, a mouse cecum was rinsed with ice-cold PBS and everted. The evetared preparation was fixed over a polyethylene tube and stirred gently for 3 h in PBS supplemented with 1 mM EDTA on ice. The isolated epithelia were collected by centrifugation (2,000 g for 20 min) and then resuspended in the PBS solution. To avoid any contamination by subepithelial cells, epithelial fragments, each with one intact crypt structure, were selected and picked up under a stereoscopic microscope. Total RNA was extracted from the mucosal-submucosal sheet and epithelia by using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RT-PCR was carried out according to the manufacturer’s instructions for the OneStep RT-PCR kit (Qiagen). The program used was one cycle of reverse transcription at 50°C for 35 min, followed by 15 min at 95°C, and 40 cycles of amplification (60 s of denaturation at 94°C, 30 s of annealing at 60°C, and 1 min of primer extension at 72°C). A final extension of 10 min at 72°C was used. The specific oligonucleotide primers for RT-PCR were as follows: PAR$_1$, 5’-CTATGAGGCAAGCCAA-3’ (sense), 5’-CTGATCTTGAAAGGGAC-3’ (antisense); and PAR$_2$, 5’-TCTGGATTTCTCTGCGGA-3’ (sense), 5’-TACGAGGAGGTTGCTAGGAC-3’ (antisense); these were derived from the published sequences for the mouse (GenBank accession nos. BC031516 and Z35158, respectively). The sizes of the expected fragments were 422 bp (PAR$_1$) and 635 bp (PAR$_2$).

**Reagents.** Bumetanide, atropine, hexamethonium, benzamil, forskolin, trypsin from porcine pancreas, and thrombin from bovine plasma were purchased from Sigma (St. Louis, MO). Tetrodotoxin (TTX) was purchased from Calbiochem (La Jolla, CA). L-703,606 and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) were purchased from Research Biochemical International (Natick, MA). Substance P was purchased from Peptide Institute (Osaka, Japan). Mouse

![Fig. 1. Changes in short-circuit current ($I_{sc}$) and transmucosal conductance ($G_t$) induced by the proteinase-activated receptor-1-activating peptide (PAR$_1$ AP). A: time-course characteristics of the changes in $I_{sc}$ and $G_t$ induced by 30 mM SFLLRN-NH$_2$ (PAR$_1$ AP) added to the serosal side at the arrowed time ($n = 18$). B: concentration dependence of the SFLLRN-NH$_2$-induced increase in $I_{sc}$ ($\Delta I_{sc}$) and $G_t$ ($\Delta G_t$). Values are the peak increases observed with each concentration of SFLLRN-NH$_2$. The number of animals used is given in parentheses.](image-url)
PAR1 AP SFFLRN-NH2 and mouse PAR2 AP SLIGRL-NH2 were respectively purchased from Yanaihara Institute (Fujinomiya, Japan) and Bachem (Bubendorf, Switzerland). Bumetanide was 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. The values are presented as the means ± SE, with n representing the number of animals. Statistical comparisons were made by Student’s paired or unpaired t-test. Significance was accepted at P < 0.05.

RESULTS

Basal electrical parameters and electrical responses to the PAR APs. The mucosal-submucosal preparation of the mouse cecum mounted in Ussing chambers exhibited an $I_{sc}$ value of 69 ± 5 μA·cm⁻² and $G_{t}$ value of 20.5 ± 0.4 mS·cm⁻² 20–30 min after the start of incubation (n = 47). The $I_{sc}$ value decreased thereafter at the rate of approximately 10% per 10 min.

PAR1 AP SFFLRN-NH2 corresponding to the tethered ligand for mouse PAR1 and PAR2 AP SLIGRL-NH2 corresponding to the tethered ligand for mouse PAR2 caused changes in $I_{sc}$ and $G_{t}$ when added to the serosal side (see the following text for details). On the other hand, peptide GYPGKF-NH2 corresponding to the tethered ligand for mouse PAR4 had no effect on either $I_{sc}$ or $G_{t}$ when added to the serosal side (up to 300 μM, data not shown) although PAR4 has been reported to be expressed in rat colonic epithelial cells (39). These three kinds of peptide failed to evoke any noticeable $I_{sc}$ and $G_{t}$ changes when applied to the luminal side (30 μM for PAR1 AP and PAR2 AP, and 300 μM for GYPGKF-NH2; n = 2 for each peptide). We did not examine the involvement of PAR3 in regulating epithelial anion secretion because there is no known peptide or agonist that specifically activates PAR3. We therefore focused on the roles of serosal PAR1 and PAR2.

Role of PAR1. Serosal SFFLRN-NH2 caused $I_{sc}$ and $G_{t}$ increases that peaked ~2 min after the addition and then decreased toward the baseline level (Fig. 1A). The $I_{sc}$ increase occurred at a concentration as low as 0.3 μM, with the maximum increase being attained at ~30 μM (Fig. 1B). The dose dependence of the $G_{t}$ increase closely resembled that of the $I_{sc}$ increase.

We first examined the ionic basis for the $I_{sc}$ increase induced by PAR1 AP (Fig. 2). Bumetanide, an Na⁺/K⁺/2Cl⁻ cotransporter inhibitor, added to the serosal side (100 μM) slightly decreased the basal $I_{sc}$ value in some, but not all, tissue samples. Pretreatment of the tissue with bumetanide significantly, but not totally, suppressed the $I_{sc}$ and $G_{t}$ increases induced by SFFLRN-NH2. When Cl⁻ was removed from both the mucosal and serosal bathing solutions, the basal $I_{sc}$ and $G_{t}$...
values were both reduced to approximately half of those under the control condition. In the absence of Cl\(^{-}\), the \(I_{sc}\) and \(G_t\) changes induced by substance P (100 nM) added to the serosal side \((n = 5)\) were both almost completely suppressed. The addition of the Cl\(^{-}\) channel/transporter inhibitor, NPPB (100 \(\mu\)M), to the mucosal side decreased basal \(I_{sc}\) but not \(G_t\), and substantially suppressed the SFFLRN-NH\(_2\)-induced \(I_{sc}\) and \(G_t\) increases. This inhibition profile was essentially similar to that observed for the \(I_{sc}\) and \(G_t\) increases induced by cAMP (serosal forskolin at 10 \(\mu\)M was used, \(n = 4\), data not shown). Because cAMP is well known to activate electrogenic Cl\(^{-}\) secretion involving the basolateral Na\(^{+}\)/K\(^{+}\)/2Cl\(^{-}\)/cotransporter and apical Cl\(^{-}\) channel (20, 37), PAR1 is likely to have stimulated similar electrogenic Cl\(^{-}\) secretion.

We next explored the role of enteric submucosal neurons in the PAR1 AP-induced Cl\(^{-}\) secretion (Fig. 3). Serosal TTX (300 nM), a nerve conduction blocker, almost completely suppressed the \(I_{sc}\) and \(G_t\) increases induced by serosal SFFLRN-NH\(_2\), indicating that the electrical responses were mediated by the submucosal neurons. It should be mentioned here that the present preparation was stripped of muscularis propria but contained largely intact submucosal nerves. TTX alone markedly suppressed basal \(I_{sc}\) (by >90\%) and significantly decreased \(G_t\) (by about 2.5 mS·cm\(^{-2}\)). We then investigated the involvement of ACh and tachykinins in the SFFLRN-NH\(_2\)-induced responses because both ACh- and tachykinin-containing neurons are present and have roles in controlling epithelial electrolyte transport in the intestines (15, 26–28, 44, 46). As summarized in Fig. 3, both the muscarinic ACh receptor antagonist, atropine (10 \(\mu\)M), and the nicotinic ACh receptor antagonist, hexamethonium (10 \(\mu\)M), partially suppressed the SFFLRN-NH\(_2\)-induced increases in \(I_{sc}\) and \(G_t\). The inhibition of \(\Delta G_t\) was only significant with the atropine treatment (Fig. 3).

Fig. 4. Changes in \(I_{sc}\) and \(G_t\) induced by substance P. 
A: time-course characteristics for the \(I_{sc}\) and \(G_t\) changes induced by substance P (100 nM) added to the serosal side \((n = 5)\). B: effects of TTX (300 nM), L-703,606 (10 \(\mu\)M), and atropine (10 \(\mu\)M) on the \(I_{sc}\) and \(G_t\) changes induced by serosal substance P (100 nM). Each inhibitor was added to the serosal side 20 min before the addition of substance P. Values for the peak increase in \(I_{sc}\) (\(\Delta I_{sc}\)) and \(G_t\) (\(\Delta G_t\)) induced by substance P were compared. The number of animals used is given in parentheses. *\(P < 0.05\) compared with the control response that was determined in the adjacent tissue using the paired \(t\)-test.

Fig. 5. Changes in \(I_{sc}\) and \(G_t\) induced by thrombin. 
Thrombin (10 U/ml) was added to the serosal side at the arrowed time \((n = 5)\).
Treatment of the tissue with L-703,606, a neurokinin-1 (NK1) receptor antagonist (10), almost completely suppressed the SFFLRN-NH2-induced increases in $I_{sc}$ and $G_{t}$ (Fig. 3). Atropine and hexamethonium had no effect on basal $I_{sc}$ and $G_{t}$, whereas L-703,606 caused a decrease in basal $I_{sc}$ (by about 40%) but not in $G_{t}$ (data not shown). These results suggest that tachykinins released from enteric neurons resulting in activation of the NK1 receptor played a major role, in addition to minor roles of ACh on the muscarinic and nicotinic receptors, in mediating the Cl⁻ secretion induced by SFFLRN-NH2. The results shown in Fig. 3 also demonstrate that the sum of the magnitude of reduction by the ACh receptor antagonists and by the NK1 receptor antagonist was more than the total $I_{sc}$ increase induced by SFFLRN-NH2, indicating that the cholinergic and tachykinergic pathways were not independent but overlapped each other.

Because the role of tachykinins in regulating epithelial transport in the mouse cecum has not been reported before, we examined the effect of substance P on $I_{sc}$ and $G_{t}$ (Fig. 4). Serosal substance P (100 nM) indeed induced increases in $I_{sc}$ and $G_{t}$, these being mainly due to activation of the NK1 receptor because they were largely suppressed by L-703,606. The substance P-induced responses were partly inhibited by TTX, suggesting that substance P was released at the neuron-
neuron junction, in addition to the neuron-epithelium junction.

The TTX-sensitive component was probably not mediated by cholinergic neurons because the substance P-induced $I_{sc}$ and $G_i$ increases were not affected by atropine.

To corroborate the functional presence of PAR1 and the SFFLRN-NH$_2$-induced Cl$^-$ secretion being the result of its activation, we examined the effect of thrombin, an activator of PAR$_1$, PAR$_3$, and PAR$_4$, but not of PAR$_2$ (40) (Fig. 5). The serosal addition of thrombin (10 U/ml) increased the $I_{sc}$ and $G_i$ values. This response was completely abolished by pretreating the tissue with TTX (300 nM) as was the response to SFFLRN-NH$_2$ ($n = 3$, data not shown). The thrombin-induced electrical response was presumably associated with its proteolytic activity because the response was markedly decreased when thrombin was heat inactivated (Supplemental Fig. S1A; supplemental material for this article is available online at the American Journal of Physiology Gastrointestinal and Liver Physiology website).

Role of PAR2. The addition of PAR2 AP SLIGRL-NH$_2$ to the serosal side caused an $I_{sc}$ change with an initially rapid peak increase (about 2 min after the addition of PAR$_2$ AP) followed by a decrease to a level below the baseline (Fig. 6A). The initially increasing component of $I_{sc}$ was apparent with more than 3 µM of SLIGRL-NH$_2$, whereas the decreasing component was apparent with 1 µM and more (Fig. 6B). SLIGRL-NH$_2$ also caused an increase in $G_i$, which was nearly sustained, at least for 20 min (Fig. 6A).

The initial phase of the $I_{sc}$ increase induced by SLIGRL-NH$_2$ was significantly inhibited by serosal bumetanide and almost completely suppressed by mucosal NPPB or Cl$^-$ removal from the bathing solution (Fig. 7). The $G_i$ increase in the initial phase was also largely inhibited under Cl$^-$-free and NPPB conditions although not significantly reduced by the bumetanide treatment. Thus the increase in $I_{sc}$ induced by SLIGRL-NH$_2$ was likely to have mainly been attributable to the activation of electrogenic Cl$^-$ secretion. In sharp contrast with the electrical responses induced by PAR$_1$ AP previously shown (Fig. 3), the $I_{sc}$ and $G_i$ increases induced by SLIGRL-NH$_2$ during the initial phase were not affected by TTX (Fig. 7, A and C).

Fig. 7. Effects of TTX (A) and bumetanide, Cl$^-$ removal, and NPPB (B) on the $I_{sc}$ and $G_i$ changes induced by PAR$_2$ AP. Mean values are presented. SLIGRL-NH$_2$ was added at the arrowed time. C: summary of the effect on the initial increases in $I_{sc}$ ($\Delta I_{sc}$) and $G_i$ ($\Delta G_i$) induced by serosal 100 µM SFFLRN-NH$_2$ of adding bumetanide (100 µM, serosal) or NPPB (100 µM, mucosal), removal of Cl$^-$ from both the mucosal and serosal bathing solutions, or TTX (300 nM, serosal). An inhibitor was added 20 min before adding SLIGRL-NH$_2$. The number of animals used is given in parentheses. *$P < 0.05$ compared with the control response that had been determined in the adjacent tissue using the paired $t$-test.
The secondary decreasing component of $I_{sc}$ induced by SLIGRL-NH$_2$ was largely suppressed by Cl$^-$/H$_2$O removal, NPPB, and TTX but not noticeably affected by bumetanide (Fig. 7). The ionic mechanism underlying this $I_{sc}$ decrease is presently not clear but may not have been attributable to the stimulation of electrogenic K$^+$/H$_2$O secretion (37) because mucosal K$^+$/H$_2$O channel blockers tetraethylammonium and Ba$^{2+}$ (10 mM and 5 mM, respectively) had no effect on the SLIGRL-NH$_2$-induced $I_{sc}$ decrease (Supplemental Fig. S2). It may not have been due to the inhibition of electrogenic Na$^+$/H$_2$O absorption (37) because the addition of epithelial Na$^+$/H$_2$O channel blocker benzamil (mucosal, 100 uM) had no effect on the baseline $I_{sc}$ value (data not shown), excluding the ongoing electrogenic Na$^+$ absorption in the cecum.

Trypsin activates PAR$_1$, PAR$_2$, and PAR$_4$, with PAR$_2$ being activated more potently than PAR$_1$ (34, 40). We therefore examined the effect of adding trypsin on $I_{sc}$ and $G_t$ to demonstrate the functional presence of PAR$_2$ (Fig. 8). Trypsin added to the serosal solution (100 uM) caused a transient increase in $I_{sc}$, followed by a decrease to a level slightly below the baseline; the time-course characteristic of $I_{sc}$ response was thus similar to the SLIGRL-NH$_2$-induced $I_{sc}$ response. Trypsin also caused an initial increase in $G_t$, but, in contrast to the SLIGRL-NH$_2$-induced $G_t$ change, this was transient and gradually decreased to a level below the baseline (Fig. 8, A and C). The initial $I_{sc}$ increase induced by trypsin was not affected by TTX but substantially suppressed by bumetanide and Cl$^-$/H$_2$O removal, whereas the initial $G_t$ increase was inhibited by Cl$^-$/H$_2$O removal but not by bumetanide or TTX (Fig. 8). The similarity between the effect on $I_{sc}$ of trypsin and PAR$_2$ AP suggests the functional presence of PAR$_2$ and that SLIGRL-NH$_2$ indeed activated PAR$_2$. The $G_t$ change observed during the second phase, however, was clearly different between trypsin and SLIGRL-NH$_2$. Trypsin may thus decrease the permeability of the paracellular pathway that is independent of PAR$_2$ activation. All of the trypsin-induced electrical responses are presumably associated with its proteolytic activity because the responses were almost abolished when trypsin was heat inactivated (Supplemental Fig. S1B).

**Stability and specificity of SFFLRN-NH$_2$ and SLIGRL-NH$_2$.** SFFLRN-NH$_2$ (300 uM), when added after the transient $I_{sc}$ increase by pretreated 100 uM SFFLRN-NH$_2$ had been completed, failed to induce an $I_{sc}$ (or $G_t$) increase (Fig. 9A), suggesting that the transient nature of the $I_{sc}$ response to SFFLRN-NH$_2$ did not result from any rapid degradation of the administered peptide but rather by the desensitization of PAR$_1$. Similarly, 30 uM SLIGRL-NH$_2$, when added after pretreatment by 100 uM SLIGRL-NH$_2$, failed to induce any change in $I_{sc}$ (or in $G_t$ on top of the sustained $G_t$ increase) (Fig. 9B), suggesting that the transient nature of the initial $I_{sc}$ response to

![Fig. 8. Effects of TTX (serosal, 300 nM) (A), and bumetanide (serosal, 100 uM) and Cl$^-$/H$_2$O removal (both serosal and mucosal) (B) on the changes in $I_{sc}$ and $G_t$ on top of the sustained $G_t$ increase) (Fig. 9B), suggesting that the transient nature of the initial $I_{sc}$ response to...
SLIGRL-NH₂ was probably attributable to the desensitization of PAR₂ rather than to any degradation of the peptide.

It has been reported that some PAR₁ agonists activated PAR₂ as well as PAR₁ (32). However, the pretreatment with SFFLRN-NH₂ did not lessen the magnitude of the increases in I_{sc} and G_{t} induced by the subsequent addition of SLIGRL-NH₂ (Fig. 9, A and C). If SFFLRN-NH₂ could activate PAR₂ and desensitize it, the electrical response to subsequently added SLIGRL-NH₂ would have been reduced. In addition, the SFFLRN-NH₂-induced I_{sc} and G_{t} increases were not reduced after PAR₂ desensitization (Fig. 9, B and C). It is thus excluded that a substantial part of the SFFLRN-NH₂-induced electrical response could have resulted from the activation of PAR₂. This notion is supported by the present finding that the I_{sc} and G_{t} increases induced by SFFLRN-NH₂ were almost completely suppressed by TTX, whereas those induced by SLIGRL-NH₂ were not affected by TTX (Figs. 3 and 7).

**DISCUSSION**

This study was designed to disclose the role of serosal PARs in regulating epithelial ion transport in the mouse cecum by using Ussing chambers and measuring the responses of I_{sc} and G_{t} to the activating peptides for each receptor. The results show that serosal PAR₁ AP and PAR₂ AP stimulated Cl⁻/H⁺ secretion although they did so in different ways; PAR₁ AP-induced secretion was mediated by the enteric nerve, whereas PAR₂ AP probably directly stimulated the epithelial cells. In addition, thrombin, an activator of PAR₁, PAR₃, and PAR₄, but...
not of PAR2 (40), and trypsin, an activator of PAR1, PAR2, and PAR4, with PAR2 being activated more potently than PAR1 (34, 40), could respectively mimic the PAR1 AP-induced and PAR2 AP-induced responses, implying that PAR1 and PAR2 were expressed in the surface membrane, where they could respond to specific proteases, thus being functionally expressed. The expression of mRNAs for PAR1 and PAR2 in the mucosa and that for PAR2 in the epithelial cells, as demonstrated by the RT-PCR results, is consistent with the conclusions drawn from the electrophysiological experiments.

The $I_{sc}$ increase in response to serosal PAR1 AP and thrombin was probably mainly due to the activation of PAR1 present on the submucosal enteric neurons because it was largely suppressed by TTX. The presence of PAR1 on submucosal neurons has previously been demonstrated functionally and immunohistochemically in the mouse proximal colon although the function there was markedly different from that in the cecum (8); in the proximal colon, PAR1 AP suppressed neurally evoked epithelial chloride secretion. The secretory response to PAR1 AP in the cecum is likely to have been mainly mediated by a release of tachykinins from enteric neurons, which then probably activated the NK1 receptor, because the response was mostly abolished by NK1 receptor antagonist L-703,606 (10). A serosal addition of the tachykinin, substance P (100 nM), indeed induced increases in $I_{sc}$ and $G_{cl}$, which were substantially attributable to activation of the NK1 receptor in the present preparation (Fig. 4). The substance P-induced responses were partly (>50%), but not totally, inhibited by TTX, suggesting the presence of the NK1 receptor on both the secretomotor neurons and epithelial cells. Consequently, NK1 involved in PAR1 AP-induced anion secretion was either neural, epithelial, or both. Tachykinins in intestinal tissues from several species are almost entirely confined to neurons, including afferent neurons, and both neurons and epithelial cells express the NK1 receptor (21, 27, 46). In addition, activation of the neuronal and epithelial NK1 receptor has been shown to stimulate Cl⁻ secretion (15, 27, 28, 46). Although myenteric neurons that express tachykinins also express PAR1 and PAR2 (16), it remains to be demonstrated whether the same combination is also the case in submucosal secretomotor and afferent neurons. PAR1 has been suggested to have a major proinflammatory role, at least under certain conditions (50). The release of tachykinins, as suggested by the results of the present study, could play a role in PAR1-mediated inflammation because tachykinins have been shown to cause intestinal inflammation via binding to the NK1 receptor (24, 46).

The present results also show that the PAR1-induced activation of Cl⁻ secretion was partially mediated by cholinergic neurons because it was partially inhibited by atropine and hexamethonium. It is known that cholinergic neurons are abundant in the intestinal tissues and that their activation leads to Cl⁻ secretion. Epithelial muscarinic ACh receptors are involved in this response (15, 26). The present results suggest that the cholinergic and tachykininergic pathways are not independent but involve some interaction. This could be due to neurons containing both ACh and tachykinins, as has been evident in other intestinal tissues (15, 21), and resulted from the synergistic stimulation of Cl⁻ secretion after being simultaneously released.

Previous immunohistochemical studies (8), as well as our RT-PCR results, demonstrate that PAR1 was also localized on epithelial cells in the mouse cecum. The activation of PAR1 in an intestinal cell line has been shown to stimulate Ca²⁺-dependent Cl⁻ secretion (7) and been implicated in the induction of apoptosis and in increased intestinal permeability (13, 14). However, an electrical response to PAR1 AP that could be attributed to an epithelial origin was hardly seen in the mouse cecum (Fig. 3, TTX). It remains to be determined whether epithelial PAR1, as demonstrated by RT-PCR, was involved in regulating an electroneutral ion transport process such as the coupled-NaCl absorption mediated by Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers (37). It is also possible that epithelial PAR1 was expressed only on the apical membrane.

The Cl⁻ secretion induced by serosal PAR2 AP and trypsin was, in contrast to that induced by PAR1 AP, not inhibited by TTX, suggesting that it was mainly attributable to the activation of PAR2 present on epithelial cells. It has been reported that SLIGRL-NH₂, which was used here as PAR2 AP, activated the NK1 receptor in a murine tracheal preparation (1). However, the involvement of NK1 in the present SLIGRL-NH₂-induced response can be excluded because the electrical response to SFLLRN-NH₂ that involved NK1 was normally elicited, even after the response to SLIGRL-NH₂ had been almost completely downregulated (Fig. 9). The expression of PAR2 in intestinal epithelial cells was demonstrated here by the RT-PCR study and has previously been reported by immunohistochemical means (18, 19, 23, 35, 43). Previous studies on the human colon and rat jejunum have shown that stimulating PAR2 caused Cl⁻ secretion in a TTX-insensitive fashion (38,
PAR2 is abundantly expressed in the submucosal neurons in a sensitive manner in the porcine ileum (23). It is known that PAR2 AP on the enteric nerve was clearly responsible for regulating intestinal ion transport in these tissues and cell lines. However, PAR2 on the enteric nerve was also involved in regulating epithelial transport because PAR2 AP has stimulated anion secretion in a TTX-sensitive manner in the porcine ileum (23). It is known that PAR2 is abundantly expressed in the submucosal neurons in the small intestine and colon and that its activation induces hyperexcitability of the neurons (18, 23, 42). Therefore, the absence of a TTX-sensitive component in the SLIGRL-NH2-induced Cl− secretion in the mouse cecum could be explained by PAR2 hardly being expressed on the submucosal secretomotor neurons if any is indeed expressed on other types of neurons in this tissue. Although neuronal mediation is unlikely, it cannot be excluded that PAR2 AP stimulated epithelial Cl− secretion indirectly through paracrine mediation by neighboring cells. These include enteroendocrine cells, myofibroblasts cells, mast cells, and other inflammatory cells that might have PARs and release secretagogues (20).

During the second phase of the electrical response induced by SLIGRL-NH2, Isc was decreased and Gi was increased, and the levels were both sustained until at least 20 min after SLIGRL-NH2 addition. The underlying epithelial mechanism for this phase is not clear. The Isc decrease attributable to the electrogenic K+ secretion has been elicited by PAR2 AP in the mouse distal colon (18), but this may not be the case in the mouse cecum, as discussed in RESULTS. The Gi increase of the second phase was possibly due to increased paracellular permeability. PAR2 activation has been reported to cause increased intestinal permeability and suggested to play a role in the pathogenesis of such diseases as ulcerative colitis and irritable bowel syndrome (5, 11–13, 30, 43).

Proteases that can activate serosal PARs and induce Cl− secretion in the intestine may arise from the lumen (endogenous and originating from intestinal flora), from the circulation system, or from inflammatory cells (5, 40, 50). These proteases are likely to be activated or recruited under a variety of pathological conditions such as intestinal infection, tissue damage, and inflammation (17, 45, 50), although luminal protease would only gain access to serosal PARs when the epithelial barrier had been compromised. The proteases thus recruited would activate serosal PAR1 and PAR2 and contribute to the induction of diarrhea under these conditions, thereby playing a role in mucosal defense as has been suggested by others (2, 50). On the other hand, whether or not PAR-mediated Cl− secretion has any physiological role in the cecum under normal conditions remains to be determined. In this context, the regulation of epithelial ion transport mediated by PARs is apparently different among the three segments of the mouse large intestine; in the cecum, PAR1 and PAR2 were both involved in the stimulation of epithelial Cl− secretion; in the mouse proximal colon, PAR1 activation led to a decrease in neurally evoked Cl− secretion (8), and, in the distal colon, PAR2 activation caused epithelial KCl secretion and inhibited electrogenic Na+ absorption (18). These findings apparently correspond to a straightforward principle: the cecum is a secretory segment for supporting bacterial fermentation; the proximal colon is an absorbing segment to recover fluid; the distal colon is a segment to adjust the body ion balance and to provide some fluid for the smooth passage of hard feces. Further studies are necessary to establish the roles of serosal PARs in each segment of the small and large intestines in healthy and diseased states.

Finally, the possible residence of epithelial PAR1, PAR2, and other PARs on the apical membrane and their role in regulating Cl− secretion or other epithelial functions need to be further explored. In this context, it would be interesting to determine whether the luminal proteases, particularly those from the bacteria that influence fermentation, have any biological or pathological roles that may or may not be mediated by PARs on the apical membrane.

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DISCLOSURES

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