Activation of proteinase-activated receptor-1 inhibits neurally evoked chloride secretion in the mouse colon in vitro

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1Mucosal Inflammation and 2Gastrointestinal Research Groups, University of Calgary, Calgary, Alberta, Canada; 3Johnson & Johnson Pharmaceutical Research and Development, Spring House, Pennsylvania; 4Dipartimento di Farmacologia Sperimentale, University of Naples; and 5Dipartimento di Chimica Farmaceutica e Tossicologica, University of Naples, Italy

Submitted 11 March 2004; accepted in final form 31 August 2004

Buresi, Michelle C., Nathalie Vergnolle, Keith A. Sharkey, Catherine M. Keenan, Patricia Andrade-Gordon, Giuseppe Cirino, Donatella Cirillo, Morley D. Hollenberg, and Wallace K. MacNaughton. Activation of proteinase-activated receptor-1 inhibits neurally evoked chloride secretion in the mouse colon in vitro. Am J Physiol Gastrointest Liver Physiol 288: G337–G345, 2005. First published September 2, 2004; doi:10.1152/ajpgi.00112.2004.—The proteinase-activated thrombin receptor-1 (PAR-1) belongs to a unique family of G protein-coupled receptors activated by proteolytic cleavage. We studied the effect of PAR-1 activation in the regulation of ion transport in mouse colon in vitro. Expression of PAR-1 in mouse colon was assessed by RT-PCR and immunohistochemistry. To study the role of PAR-1 activation in chloride secretion, mouse colon was mounted in Ussing chambers. Changes in short-circuit current (Isc) were measured in tissues exposed to either thrombin, saline, the PAR-1-activating peptide TFLLR-NH2, or the inactive reverse peptide RLLFT-NH2, before electrical field stimulation (EFS). Experiments were repeated in the presence of either a PAR-1 antagonist or in PAR-1-deficient mice to assess receptor specificity. In addition, studies were conducted in the presence of chloride-free buffer or the muncarinic antagonist atropine to assess chloride dependency and the role of cholinergic neurons in the PAR-1-induced effect. PAR-1 mRNA was expressed in full-thickness specimens and mucosal scrapings of mouse colon. PAR-1 immunoreactivity was found on epithelial cells and on neurons in submucosal ganglia where it was colocalized with both VIP and neuropeptide Y. After PAR-1 activation by thrombin or TFLLR-NH2, secretory responses to EFS but not those to forskolin or carbamol were significantly reduced. The reduction in the response to EFS was not observed in the presence of the PAR-1 antagonist, in PAR-1-deficient mice, when chloride was excluded from the bathing medium, or when atropine was present. PAR-1 is expressed in submucosal ganglia in the mouse colon and its activation leads to a decrease in neurally evoked epithelial chloride secretion.

Enteric nervous system; epithelial ion transport; thrombin; submucosal secretomotor neurons

Proteinase-activated receptors (PARs) are a recently characterized class of G protein-coupled receptors activated by the proteolytic cleavage of their NH2 termini by specific serine proteinases. The new NH2 terminus acts as a “tethered ligand” that binds to other extracellular domains of the receptor to initiate G protein-dependent signaling. PAR-1, the prototypical thrombin receptor, was the first of four PARs to be cloned to date (21, 26, 43, 44). PAR-1 is found in diverse cell types including epithelial cells, platelets, endothelial cells, fibroblasts, monocytes, T-cell lines, osteoblast-like cells, smooth muscle cells, neurons, and glial cells, and in certain tumor cell lines (8, 14, 26). Given the wide tissue distribution of PAR-1, it is likely that this receptor plays an important role in a variety of cellular functions.

Involvement of thrombin in inflammation is well described (12), and its participation, via activation of PAR-1, has been demonstrated in platelet aggregation, vasodilatation, increased vascular permeability, and granulocyte chemotaxis (15). Thrombin has been implicated in various aspects of the pathogenesis of inflammatory bowel disease (IBD) (11, 35, 39). Although thrombin levels have not been directly measured in the intestinal lamina propria of patients with IBD, Crohn’s disease and ulcerative colitis are associated with a thrombotic tendency, which may contribute to the clinical features of this disease (19, 39). Patients with Crohn’s disease also show various coagulation abnormalities, and intestinal vascular injury, such as intestinal microinfarction, has been proposed as a major pathogenic factor (10, 38, 45).

Due to the vascular disruption and the increased microvascular permeability characteristic of inflamed tissues, it is likely that active thrombin would be in a position to affect many cell types in the mucosa of the inflamed gut, including the epithelium. The intestinal epithelium represents the first line of defense against luminal bacteria, bacterial products, and food antigens. This defensive role is carried out, in part, through the ability of a subpopulation of enterocytes to secrete chloride, which promotes the movement of water in a basolateral-to- apical direction. Secretion of fluid into the lumen of the intestine serves, among other functions, to flush away potentially harmful luminal contents and thus slows or prevents the translocation of bacteria and antigens into the lamina propria of the intestine (2, 47). The disruption of secretory function is characteristic of models of intestinal inflammation (2, 17, 27) and is observed in human IBD (1, 22, 33). The altered regulation of secretory function may in turn contribute to inflammatory conditions of the gut (2, 3).

We have previously described (7, 8) the prosecretory effects of PAR-1 activation in an intestinal epithelial cell line. However, PAR-1 is also expressed in cell types that regulate epithelial secretory function, and the role of thrombin and PAR-1 in the integrated regulation of chloride secretion in intact intestinal tissue has not yet been studied. Given the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The important role of thrombin in inflammation, elucidating the involvement of PAR-1 in the secretory dysfunction associated with inflammatory conditions of the gut is of potential clinical importance. Thus, we sought to determine the effects of PAR-1 activation on the regulation of ion secretion in intact tissue in mouse colon in vitro.

MATERIALS AND METHODS

Animals

Male wild-type C57BL6 mice (Charles River, Montreal, Canada) and PAR-1-deficient mice (C57BL6 background, backcrossed for 8 generations and obtained from Johnson and Johnson Pharmaceutical Research and Development, Spring House, PA) were used in these studies. All mice were 4–6 wk of age at the time of arrival and were housed at a constant photoperiod (12:12-h light-dark cycle) and temperature (22°C). Mice were allowed to acclimatize to these conditions for a minimum of 5 days before inclusion in experiments and were allowed free access to tap water and standard lab chow. All procedures involving the mice were approved by the University of Calgary Animal Care Committee, and experiments were conducted in accordance with the guidelines established by the Canadian Council on Animal Care.

RT-PCR

RT-PCR was conducted as previously described (27) to determine the expression of PAR-1 mRNA in mouse colon. Expression of the housekeeping gene GAPDH was assessed as an internal control. Mice were euthanized by cervical dislocation and 20-mg samples of either full-thickness sections or mucosal scrapings were immediately excised, frozen in liquid nitrogen, and stored at −80°C. RNA was isolated from samples using the RNeasy method carried out according to the manufacturer’s instructions (Qiagen, Missisauga, ON). Briefly, tissue samples were thawed and homogenized in the lysis buffer provided with the kit. Samples were centrifuged at 20,000 g for 3 min and the supernatants were combined with equal volumes of 70% ethanol. Samples were then loaded onto RNeasy spin columns and were RNA purified by using a series of 15-s spins (9,000 rpm) with wash buffers provided in the kit. RNA was finally eluted from columns in 30 μl of ultrapure diethyl pyrocarbonate (DEP)-treated water (Invitrogen, Burlington, Ontario).

The purity and concentration of the isolated RNA were determined using a GeneQuant II nucleic acid analyzer (Pharmacia Biotech, Uppsala, Sweden). RNA (2 μg) was added to a reaction mixture containing 2 μl of 10× PCR buffer, 2 μl of 10 mM deoxynucleotide triphosphates (dNTPs), 2 μl of 900 mmol/μl random hexamers (Ne), and 0.5 μl of RNAguard (Invitrogen). Superscript enzyme (300 U; Gibco-BRL, Manassas, VA) was added for reverse transcription. RNA samples were first incubated for 10 min, and the reaction mixture was heated to 42°C for 50 min and then to 95°C (to destroy the Superscript enzyme) in a DNA Engine Thermal Cycler (MJ Research, Waltham, MA). cDNA obtained from this reaction was then cooled to 4°C and stored at −20°C. PCR was performed on the cDNA in a Peltier Thermal Cycler (model PTC-200; MJ Research, Waltham, MA). The reaction mixture contained 5 μl of 10× PCR buffer, 1 μl of each of the 5’ and 3’ primers, 0.5 μl of HotStarTaq DNA polymerase (Qiagen), 2 μl of the cDNA template, and 38 μl of DEP-treated H2O (Invitrogen). The primer sequences used for PAR-1 amplification were 5’-ATGAGGGCCGCGGGCC-3’ and 5’-GGCTTGTCAGATACCGGAA-3’, whereas those used for GAPDH amplification were 5’-CGGAGTCAACGAGATTGGTCGTAT-3’ and 5’-AGCCTTCTGCTGTTCAAGAC-3’ (27). PCR for PAR-1 was stopped after 42 cycles (denaturation at 94°C for 1 min, annealing at 54°C for 1 min and elongation at 72°C for 1 min). PCR for GAPDH was performed under the same conditions and was stopped after 38 cycles. Samples from the PCR were separated on a 1% agarose gel containing ethidium bromide for 5 min at 100 V and 40 min at 80 V. The gel was then photographed by using a Gel Doc 2000 (Bio-Rad, Hercules, CA).

Assessment of Chloride Secretion

For the measurement of PAR-1 regulation of chloride secretion, mouse colon was excised, opened along the mesenteric border, cleaned with cold (4°C) Krebs buffer, and mounted in Ussing chambers exposing 0.6 cm2 of the mucosal surface. Tissues were bathed on both sides with Krebs buffer (pH 7.4, 37°C) containing 10 mM glucose. Krebs buffer contained (in mM): 115 NaCl, 2.0 K2HPO4, 2.4 MgCl2, 25 NaHCO3, 8 KCl, and 1.3 CaCl2. Buffers were aerated and circulated with a gas lift system (5% CO2-95% O2). In some experiments, tissues were instead bathed on either side with a buffer lacking chloride. This buffer contained (in mM) 117 sodium isethionate, 2 K2HPO4, 2.4 hemimagnesium gluconate, 25 NaHCO3, 8 potassium gluconate, and 10 glucose, plus 650 μM calcium gluconate. The transepithelial potential difference was clamped to 0 V by applying a short-circuit current (Isc) with a voltage-clamp apparatus (EVC-4000, World Precision Instruments, Sarasota, FL). Voltage was measured, and Isc was delivered via two pairs of glass-barreled, ceramic-tipped Ag-AgCl electrodes filled with 3 M KCl, with one member of each pair located on either side of the tissue. The changes in Isc (ΔIsc) were an indicator of changes in the net electrogenic electrolyte flux across the tissue. Isc was recorded with a digital data acquisition system (model MP100; BioPac, San Diego, CA) and analyzed with AcqKnowledge software (version 3.2.6; BioPac). All measurements were normalized to 1 cm2 surface area.

PAR-1 activation was accomplished with the serosal addition of either thrombin (5 U/ml) or the PAR-1-activating peptide TFLLR-NH2 (50 μM) (24). These concentrations were based on those used to give near maximal responses in cell line studies (24). Near maximal concentrations were used to ensure diffusion across the full-thickness mouse colonic segments. Concentration-response curves were not constructed because of peptide cost and the large amounts required in our Ussing chamber baths. Saline and the inactive reverse peptide RLLT-NH2 (50 μM) were used as controls. It was necessary to use an inactive peptide as a control to distinguish between the nonspecific peptide effects and PAR-1-specific effects. Previous studies (41) have shown nonspecific biological activity, in terms of inflammation, of some PAR-activating peptides. Electrical field stimulation (EFS; 50 V, 10 Hz, 500-μs pulse duration, 5-s total stimulus duration) was applied 5 min after administration of PAR-1 agonist or control, followed by serosal application of the secretagogues carbachol (10 μM), which activates calcium-dependent secretion and forskolin (10 μM), which activates cAMP-dependent secretion. Isc was allowed to return to baseline levels between each stimulus. The muscarinic antagonist atropine (1 μM), when used, was administered at the same time as PAR-1 agonist or control and was added to the buffers bathing both the serosal and mucosal sides of the tissue. PAR-1 antagonist (100 μM) was added to the serosal side only, 5 min before administering PAR-1 agonist or control.

Immunohistochemistry

Cross sections. Proximal colon from wild-type and PAR-1-deficient mice was excised and fixed in Zamboni’s fixative (2% paraformaldehyde, 15% picric acid) for 18 h and washed in PBS (0.55 M K2HPO4, 0.1 M NaH2PO4·H2O, 0.31 M NaCl) with 0.01% (wt/vol) sodium azide. Samples were cryoprotected overnight in PBS containing 20% sucrose, embedded in optimal cutting temperature compound (Sakura Finetek, Toland, CA) and placed on Superfrost Plus microslides (VWR Scientific, West Chester, PA). Slides were incubated for 30 min with blocking solution consisting of goat serum.
diluted to a concentration of 1:10 in antibody diluent (PBS containing 7.7 mM sodium azide, 1.1 mM sodium EDTA, 0.1% bovine serum albumin, and 10% Triton X-100). Slides were then stained overnight at 4°C with a rabbit polyclonal antiserum derived against an amino acid sequence that spans the thrombin cleavage site (YATPNPRSF-FLRNPSED) in the NH₂ terminus of rat PAR-1. The antiserum was used at a concentration of 1:250 in antibody diluent. As a negative control, PAR-1 antiserum was diluted in antibody diluent and incubated overnight at 4°C with a blocking peptide (corresponding to the antigen used for immunization and synthesized in house, a final concentration of 1:250 for the antibody, and 40 μg/ml for the blocking peptide) before being applied to sections. Slides were then washed 4 × 10 min with PBS containing 0.01% sodium azide and 0.1% Triton X-100. Sections were then incubated at room temperature for 90 min with CY3-conjugated donkey anti-rabbit IgG antibody diluted 1:100 in antibody diluent. Slides were washed four more times with PBS containing azide and Triton X-100 and then examined by using a Zeiss Axioplan fluorescence microscope. Images were captured with a digital camera (Sensys, Photometrics; Tucson, AZ) using V for Windows image analysis software (Digital Optics, New Zealand).

Whole mounts. Proximal colon was removed from mice after cervical dislocation and opened along the mesenteric border. Tissues were rinsed in PBS and placed in cold PBS containing 1 μM nifedipine for 10 min before being stretched and pinned mucosal side up in a Sylgard-coated petri dish. The preparation was incubated in Zamboni’s fixative at 4°C overnight and then washed 2 × 30 min with PBS. To isolate the submucosal plexus, preparations were pinned flat with the mucosal surface facing upward in a Sylgard-coated petri dish. Mucosa was removed by gentle strokes with fine forceps and discarded. The submucosal plexus was separated from the underlying muscle by delicate peeling.

Isolated segments of submucosal plexus were placed in separate wells of a 48-well plate. Blocking solution (normal goat serum in PBS, 1:10) was added to the wells and left on for 60 min before being replaced with antibody solutions diluted in antibody diluent. Primary antibodies were left on for 48 h, at 4°C, and then washed 3 × 10 min with PBS containing 0.01% sodium azide and 0.1% Triton X-100. Tissues were incubated in secondary antibody for 90 min and washed three more times before being mounted in bicrobanate-buffered glyceral. Samples were visualized as described above. To establish whether PAR-1 colocalized with cholinergic or VIP-containing neurons, whole-mount preparations were double labeled using the PAR-1 antiserum and antibodies directed against either neuropeptide Y (NPY) or against VIP. Staining for PAR-1 and NPY was conducted simultaneously, whereas staining for VIP was performed by using a modified protocol for a mouse-on-mouse (MOM) immunodetection kit (Vector Laboratories, Burlingame, CA) after PAR-1 staining. Briefly, tissues were incubated 1 h in MOM Ig block agent, washed 2 × 2 min in PBS, and incubated with primary antibody (mVIP31, 1:100) in MOM antibody diluent for 48 h. Tissues were washed 2 × 2 min in PBS and incubated 1 h with MOM biotinylated anti-mouse Ig reagent for 60 min, followed by washing 2 × 2 min in PBS and incubation with fluorescein-avidin colorimetric substrate for 60 min. Tissues were then washed and mounted as described above. The antibodies used for detection of PAR-1, VIP, and NPY immunoreactivity are summarized in Table 1.

### Materials

All buffer components were from either BDH (Toronto, Canada), Sigma-Aldrich (St. Louis, MO), or EM Sciences (Gibbstown, NJ). PCR buffer was from Amersham Biosciences (Piscataway, NJ), and RNA guard, N₆, and dNTPs were purchased from GIBCO (Burlington, Canada). Forskolin, carbachol, atropine, and Triton X-100 were all from Sigma. MSPO was from Boehringer-Mannheim (Mannheim, Germany). The PAR-1-activating peptide TFLLR-NH₂ and the reverse peptide RLLFT-NH₂ were synthesized in house as carboxy-amides by the University of Calgary Peptide Synthesis Facility. Thrombin (human, specific activity 2800 NIH U/mg protein) was from Calbiochem (La Jolla, CA). The PAR-1 antagonist, 4-methoxy-N-1-[(2,6-dichlorophenyl)methyl]-1H-indol-6-yl-aminocarbonyl]-L-phenylalanyl-N-(phenylmethyl)-L-arginamide, was prepared in the Dipartimento di Chimica Farmaceutica e Tossicologica, University of Naples, and has previously been reported (16).

### Statistics

Each group consisted of >5 animals. Data are presented as means ± SE. Comparison of more than two groups was made by using ANOVA with a post hoc Tukey’s test by using Instat version 3.00 software (GraphPad Software, San Diego, CA). Comparison of two groups was made by using Student’s t-test for unpaired data. An associated P value of <0.05 was considered significant.

### RESULTS

**PAR-1 Expression in Mouse Colon**

PAR-1 mRNA was detected by RT-PCR in both full-thickness and mucosal scrapings of mouse colon (Fig. 1). PAR-1 immunoreactivity could be detected in cross sections of mouse colon, predominantly on surface and crypt epithelial cells (Fig. 2A). Preincubation with the neutralizing peptide resulted in markedly diminished PAR-1 immunoreactivity (Fig. 2B). Immunoreactivity was not observed in PAR-1-deficient mice (Fig. 2C).

![Fig. 1. Reverse image of RT-PCR gel for proteinase-activated receptor-1 (PAR-1) from mouse colon. PAR-1 mRNA expression was observed in both full-thickness specimens of mouse colon and mucosal scrapings.](http://ajpgi.physiology.org/)
Measurement of Net Electrogenic Ion Flux

When PAR-1 agonist (thrombin 5 U/ml or TFLLR-NH₂ 50 μM) was added to either serosal or mucosal buffers bathing the segment of mouse colon, no change in \( I_{sc} \) was observed. EFS elicited a transient increase in \( I_{sc} \). Preparations treated serosally with either thrombin or the PAR-1 agonist TFLLR-NH₂ had significantly reduced responses to EFS compared with those observed in tissues treated with either saline or 50 μM of the inactive reverse peptide RLLFT-NH₂ (Fig. 3). PAR-1 activation affected only the amplitude of the response to EFS. It did not change the duration of the response (data not shown). Subsequent administration of the cholinergic agonist, carbachol, or of the activator of adenylate cyclase, forskolin, also caused a transient increase in \( I_{sc} \) in all preparations. Responses to carbachol and forskolin were not different in tissues treated with PAR-1 agonists compared with controls (Fig. 3).

To determine the ionic basis for the inhibitory effect of PAR-1 activation on the response to EFS, the experiments represented in Fig. 3 were repeated in chloride-free buffer. The intention of this experiment was to compare the effect of PAR-1 agonist (thrombin or PAR-1-activating peptide) vs. control (vehicle or reverse peptide, respectively) on the response to EFS in the absence of chloride. Under these conditions, the responses to all of the stimuli were markedly diminished (Fig. 4) compared with the \( I_{sc} \) responses elicited in buffer containing chloride (Fig. 3). In the absence of chloride, PAR-1 agonism no longer resulted in a reduced response to EFS, compared with that elicited in tissues pretreated with the controls. Responses to carbachol and forskolin were also not different between treatment groups (data not shown).

To assess whether the effects of thrombin and TFLLR-NH₂ on the response to EFS were indeed due to activation of PAR-1, a specific antagonist of this receptor was added to the serosal buffer (containing chloride) and the above experiments were repeated. In some tissues, addition of the antagonist caused an increase in the \( I_{sc} \), but this was not a consistent observation (data not shown). In the presence of the antagonist, the response to EFS was not different after pretreatment with thrombin or TFLLR-NH₂ compared with that seen after pretreatment with saline or RLLFT-NH₂, respectively (Fig. 5).

To further determine the role of PAR-1 in neurally evoked ion transport, studies were repeated in PAR-1-deficient mice.

![Fig. 2](http://ajpgi.physiology.org/)

Fig. 2. Micrographs of cross sections of mouse colon stained with an antibody raised against PAR-1. A: positive immunoreactivity was observed in the epithelium with the most intense staining occurring in surface epithelial cells (arrowheads). B: loss of immunoreactivity in preparations in which the primary antibody was preabsorbed with the sensitizing peptide. C: lack of immunoreactivity in PAR-1-deficient mice. Each micrograph was taken with the same exposure settings. Scale bar, 50 μm.

![Fig. 3](http://ajpgi.physiology.org/)

Fig. 3. Effect of PAR-1 activation on short-circuit current responses (Δ\( I_{sc} \)) of unstripped segments of mouse colon to electrical field stimulation (EFS), 10 μM carbachol (CCH), and 10 μM forskolin (FSK). A: PAR-1 was activated with 5 U/ml of thrombin. Saline was used as the control. B: PAR-1 was activated with the selective activating peptide TFLLR-NH₂ (50 μM). The inactive reverse-sequence peptide RLLFT-NH₂ (50 μM) was used as control. *P < 0.05 vs. control.

![Fig. 4](http://ajpgi.physiology.org/)

Fig. 4. Response to EFS in unstripped segments of mouse colon mounted in Ussing chambers. Tissues were bathed in chloride-free buffer and exposed to thrombin (5 U/ml), saline, TFLLR-NH₂ (50 μM), or the control peptide RLLFT-NH₂ (50 μM).
Interestingly, PAR-1-deficient mice had a significantly lower baseline $I_{sc}$ than wild-type controls (Fig. 6). However, there was no significant difference in the $I_{sc}$ response to EFS in PAR-1-deficient mice compared with that in wild-type controls (Fig. 6). In fact, the response to EFS in PAR-1-deficient mice appeared to be larger, but this apparent increase did not reach statistical significance.

To examine whether cholinergic secretomotor neurons were mediating the inhibitory effect of PAR-1 activation on neurally evoked ion transport, experiments were conducted in the presence or absence of the muscarinic antagonist atropine (1 $\mu$M). Administration of atropine in conjunction with either saline or RLLFT-NH$_2$ resulted in a significantly reduced response to EFS compared with the responses observed in the presence of saline or RLLFT-NH$_2$ alone. When atropine was administered in conjunction with thrombin or TFLLR-NH$_2$, the response to EFS was not further reduced compared with the response elicited in the presence of atropine and the controls (Fig. 7). Attempts were made to determine whether the inhibitory effect of PAR-1 activation on the response to EFS was also dependent on the release of VIP by enteric neurons. VIP$_6$–28, a competitive antagonist of the VIP receptor (Sigma) was applied to the buffer on either side of the tissue; however, in our hands, the response to subsequent serosal application of VIP (Sigma) itself was not consistently blocked (data not shown). Furthermore, when repeated applications of VIP were performed in an attempt to desensitize VIP receptors, the initial response to VIP in our preparations was inconsistent and at times elicited no change in $I_{sc}$ (data not shown). It was thus not possible to determine unequivocally whether receptor desensitization had occurred, and further examination of the role of VIP in PAR-1-mediated effects was not pursued.

**Localization of PAR-1 with NPY- and VIP-Containing Neurons**

In whole-mount preparations of the submucosa, PAR-1 immunoreactivity was found in the majority of neurons of the submucosal plexus (estimated to be 70–80%, Figs. 8 and 9). Immunoreactivity was distributed in the cytoplasm, was largely perinuclear, and was absent when the primary antibody was preabsorbed with the sensitizing fragment of the PAR-1 protein (Fig. 8). It was apparent that many immunoreactive...
PAR-1 expression has previously been demonstrated in various cell types in the gastrointestinal tract, including smooth muscle cells, myenteric neurons (18, 23), endothelial cells of the lamina propria and submucosa (46), and epithelial cell lines as we have previously demonstrated (8). In the present study, we demonstrated, by using RT-PCR, that PAR-1 is expressed in mouse colon, including the mucosa. PAR-1 expression on epithelial cells of the mouse colon was shown by immunofluorescence. Studies using an antibody-neutralizing peptide, as well as PAR-1-deficient mice, were carried out to assess the specificity of PAR-1 staining. PAR-1 immunoreactivity was virtually abolished in PAR-1-deficient mice and by preincubation of the antibody with neutralizing peptide, suggesting that the immunoreactivity observed is indeed a specific indication of PAR-1 expression and distribution. The presence of PAR-1 in mouse colonic epithelium is in accordance with our observation of PAR-1 expression in a human intestinal epithelial cell line (8) and suggests that direct activation of PAR-1 on enterocytes in mouse colon might evoke ion transport. However, we also observed PAR-1 protein expression in whole-mount preparations of submucosal plexus, suggesting that other cell types, specifically neurons, are potential targets for PAR-1 agonists to alter epithelial secretory function. Thus, it was imperative that we investigate the net effect of PAR-1 agonists on ion transport in whole-thickness segments of mouse colon.

The ability of enterocytes to secrete electrolytes and water into the intestinal lumen represents a critical feature of mucosal defense and may be influenced by endocrine factors, as well as by mediators released from neighboring cells, such as nerve and immune cells. The role of PARs in the regulation of intestinal secretory function is not fully understood nor have underlying mechanisms been clearly elucidated. PAR-2, which is activated by trypsin and tryptase, has been shown to modulate intestinal ion transport in several systems. For instance, activation of the receptor in the mouse distal colon stimulates Cl− and K+ secretion while inhibiting baseline Na+ absorption (13). PAR-2 is expressed in human colonic epithelium and mediates intestinal electrolyte secretion induced by the release of mast cell tryptase (28). It has also been suggested that PAR-2 may have an important role in intestinal neuroimmune modulation. Tryptase derived from mast cells can activate PAR-2 and induce long-term hyperexcitability of guinea pig submucosal neurons (31), raising the possibility that the action of this proteinase at PAR-2 could thus alter secretory function. Indeed, cholinergic and noncholinergic submucosal neurons in porcine ileum have been shown to express PAR-2, and agonists of the receptor stimulate active anion secretion by a neurogenic mechanism (20).

To investigate the role of PAR-1 in the alteration of the ability of the epithelium to respond to other secretory stimuli, mouse colon was mounted in Ussing chambers under IiC conditions. When mouse colon was exposed to either serosal or mucosal application of PAR-1 agonists, no change in IiC was observed, despite the apparent epithelial localization of PAR-1 on colonic epithelial cells. Several possible interpretations arise from this finding. PAR-1 activation may have no effect on basal ion transport in mouse colon or it may be affecting only electroneutral ion fluxes that would not be detected by the Ussing chamber technique. Alternatively, PAR-1 may be expressed on a subpopulation of epithelial cells distinct from that

**DISCUSSION**

Our previous studies (7, 8) showed that PAR-1 activation caused chloride secretion in the SCBN intestinal epithelial cell line, but the role of this receptor in secretory function had not been explored in intact intestinal tissue or in an animal model. An integrative approach is important, given the variety of cell types known to regulate epithelial secretory function. In the present study, we show that in the mouse colon, PAR-1 activation plays an inhibitory role in the chloride secretory response to neural stimulation, likely through inhibition of cholinergic secretomotor neurons.
responsible for chloride secretion. Whereas we have shown PAR-1-stimulated chloride secretion in SCBN cells (8), it should be noted that this is a duodenal cell line (30) and may not be representative of murine colonic epithelium. Finally, the lack of a direct effect of PAR-1 agonists on ion transport in mouse colon may have been due to a lack of penetration of the activators to the level of the epithelium, although the data clearly indicate that they diffused as far as the submucosal plexus. Attempts to conduct experiments in segments of colon stripped of external muscle, which presumably would have removed a major barrier to diffusion, were unsuccessful because the stripping process damaged the mucosa, rendering the tissue unresponsive to secretagogues.

After application of PAR-1 agonist or controls to the serosal buffer, the $I_{sc}$ responses to several secretory stimuli were measured in the Ussing chambers. It was observed that in the presence of PAR-1 agonist, the $I_{sc}$ response to EFS was significantly reduced compared with the response elicited in the presence of saline or the control peptide RLLFT-NH$_2$. In contrast, the secretory responses to carbachol and forskolin were not altered by the application of PAR-1 agonist. This suggests that PAR-1 activation specifically inhibits neurally evoked secretory responses, as opposed to those elicited by direct stimulation of the epithelium. Having made this observation, we then sought to determine the ionic mechanism and PAR-1 specificity of the response.

Luminally directed chloride secretion has been shown to underlie the bulk of the $I_{sc}$ response, although other processes, such as bicarbonate ion secretion, also contribute to the overall increase in $I_{sc}$ (9). To determine which of these processes was being affected by PAR-1 activation to result in a decreased response to EFS, experiments were carried out in chloride-free buffer. Under these conditions, PAR-1 activation no longer resulted in a reduced secretory response to EFS, suggesting that the inhibitory effect of PAR-1 activation was due to an effect on chloride secretion.

A PAR-1 antagonist was used to confirm that the inhibitory effects of thrombin or TFLLR-NH$_2$ application on the response to EFS were indeed due to activation of PAR-1. In the presence of the antagonist, the secretory response to EFS was unaltered by PAR-1 activation. In some tissues, serosal application of the PAR-1 antagonist itself resulted in an increase in $I_{sc}$, although this was not a consistent observation. It may suggest a role for PAR-1 in the tonic suppression of basal electrogenic ion transport, a contention supported by our observation that basal $I_{sc}$ was significantly increased in colons from PAR-1-deficient mice. The smaller overall responsiveness of the tissue in these experiments was likely due to the intergroup seasonal variations we see in epithelial ion transport (W. K. MacNaughton, unpublished observations).

We sought to determine whether the inhibitory effect of PAR-1 activation on neurally evoked chloride secretion was mediated by noncholinergic VIP or cholinergic populations of secretomotor neurons. Our immunohistochemical data support the view that noncholinergic neurons were the likely target of PAR-1 agonists, because we found that the majority of PAR-1 immunoreactive neurons expressed VIP, which to date has been considered a marker of noncholinergic secretomotor neurons (5). It is estimated that ~20% of submucosal neurons in the mouse colon are cholinergic (34), and, on the basis of their

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Fig. 9. Fluorescence micrographs of PAR-1 (A and D), VIP (B), and neuropeptide Y (NPY; E) and overlaid images of PAR-1/VIP (C) and PAR-1/NPY (F) immunoreactivity in the submucosal plexus of the mouse colon. The majority of PAR-1 was colocalized with VIP (C), although occasional NPY immunoreactive neurons also expressed PAR-1 (F). Single arrowheads show double-labeled neurons. Double arrowheads illustrate neurons not PAR-1 immunoreactive but immunoreactive for either VIP or NPY. In A–C, all PAR-1 neurons were colocalized with VIP immunoreactivity. Scale bar, 50 μm.
distribution, it seems likely that these, too, will coexpress NPY (and not VIP) as a unique marker, although this has yet to be shown.

Our contention was that, if PAR-1 on secretomotor neurons was activated and the receptors of the secretory neurotransmitter released by these neurons were concomitantly blocked, then the PAR-1-mediated inhibition of the response to EFS would be of the same magnitude as that seen without neurotransmitter receptor blockade. To block the activity of cholinergic neurons, atropine was applied in conjunction with thrombin, saline, TFLLR-NH₂, or RLLFT-NH₂. In the presence of atropine and either saline or RLLFT-NH₂, the response to subsequent stimulation with EFS was significantly lower than that observed in the presence of saline or RLLFT-NH₂ alone, as expected. In the absence of atropine and a PAR-1 activator, however, the response to subsequent EFS was not different from that seen in the presence of atropine alone. The lack of an additive effect of PAR-1 activation and atropine suggests that at least part of the inhibitory effect of PAR-1 activation is through inhibition of cholinergic neurons. Nevertheless, due to the experimental difficulties we encountered in blocking VIP receptors, it cannot be concluded with certainty that VIPergic neurons are not also involved. How the relatively small population of PAR-1-expressing neurons contributes to the inhibition of secretion is not clear. It is unlikely that epithelial PAR-1 contributes to decreased secretion by inhibiting muscarinic signaling at the level of the epithelial cell, because PAR-1 activation did not affect the response to carbachol, which stimulates epithelial muscarinic receptors to stimulate secretion. Several explanations are possible. PAR-1 activation could (i) prevent release of acetylcholine from the cholinergic secretomotor neuron, (2) prevent acetylcholine reuptake mechanisms, or (3) stimulate release of an inhibitory neurotransmitter that could block neurally mediated chloride secretion. More studies will be necessary to elucidate which of these mechanisms is responsible for our observations.

The endogenous activator of neuronal PAR-1 is not known. Although there is no evidence to date for the presence of thrombin in cells of the enteric nervous system, this protease has been detected in astrocytes of the central nervous system under some conditions (6). Because enteric glia resemble astrocytes (32), these cells could potentially be a source of thrombin in the enteric nervous system. Thrombin and PAR-1 may thus mediate certain neuronal functions under physiological or pathophysiological conditions. Given the usual association of thrombin with inflammatory states, it may be that circulating, activated thrombin released during inflammation-associated microvascular leakage exerts an effect on enteric neuronal PAR-1. However, it is possible that another protease is responsible for the constitutive PAR-1 activation. The gastrointestinal tract is exposed to a particularly large array of proteases, whether in physiological or pathophysiological settings, due to its proximity to digestive enzymes, proteases from pathogens, and proteases derived from inflammatory cells, or the microvasculature (reviewed in Ref. 40). Plasmin, granzyme A, and cathepsin G, as well as some bacterial proteases have been shown to cleave and activate PARs, with varying potency (25, 29, 36, 37, 42). Thus, in many tissues, including the colon, the identities of proteases that stimulate PARs in vivo require further clarification (4).

In conclusion, we have demonstrated that in the mouse colon, PAR-1 activation leads to a decreased secretory responsiveness to neural stimulation. This appears to be associated with a decreased net fluid flux into the lumen of the intestine. The function of PAR-1 expressed on the epithelium remains to be established. Findings of this study have broad implications for the function of chloride-transporting epithelia, which may, in the case of ongoing inflammation, be exposed to active thrombin.

REFERENCES

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