Activation of ion secretion via proteinase-activated receptor-2 in human colon

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SERINE PROTEASES HAVE RECENTLY been demonstrated to act as signaling molecules (12). They regulate cells by specifically cleaving and activating members of a new family of protein-activated receptors (PARs). Apart from the three types of thrombin receptors, PAR-1, -3, and -4, the subtype PAR-2 has been identified as a receptor for trypsin and mast cell tryptase (12, 27). PARs are G protein-coupled receptors that are activated either by soluble ligands that reversibly bind to the receptor or by irreversible cleavage by a protease.

The function and physiological significance of PAR-2 is still poorly understood. However, recent studies suggested that PAR-2 might participate in the control of ion transport in gastrointestinal epithelia. Thus PAR-2 has been implicated in activating ion transport in cultured dog pancreatic duct cells (26). These studies suggested an activation of luminal Cl− and basolateral K+ channels in pancreatic epithelial cells due to stimulation of basolaterally located PAR-2. Such an activation of ion secretion may promote clearance of toxins and debris from the pancreatic duct. Thus pancreatic trypsin may activate PAR-2, which are located in the pancreatic duct under both physiological and pathological conditions. PAR-2 have also been identified in both luminal and basolateral membranes of epithelial cells in the small intestine (21). Expression of these receptors has been demonstrated by Northern blot analysis and immunohistochemistry (10, 21), and previous data obtained from rat jejunal mucosa suggested activation of ion transport by trypsin (33). On the basis of considerable differences in agonist concentrations and potency profiles for activation of short-circuit currents, the authors concluded that a receptor...
different than PAR-2 is activated by trypsin in the rat intestine (33).

However, the nature of ion conductances that are activated by stimulation of intestinal PARs and the responsible intracellular second-messenger pathways are still largely unknown. In the present study, we explored these questions by using a modified Ussing-chamber technique, which allows measurement of ion transport in small human rectal biopsies. The results presented here indicate pronounced activation of ion transport in the human colon by trypsin. Electrolyte secretion could be elicited repeatedly by trypsin in the same mucosal biopsy. It is thus very likely that activation of PARs, present in basolateral membranes of human colonic epithelial cells, contributes to the hypersecretion found in inflammatory processes such as chronic inflammatory bowel disease (13).

MATERIALS AND METHODS

Patients. Ussing-chamber measurements were performed on rectal mucosa biopsies obtained from 30 normal individuals and 11 cystic fibrosis (CF) patients. All CF subjects presented with pancreatic insufficiency and chronic lung disease and fulfilled the diagnostic criteria of CF, including elevated sweat tests (30). Genotyping by DNA analysis of all CF patients showed that five were homozygous and six were heterozygous for ΔF508-CF transmembrane conductance regulator (CFTR; allelic frequency of 72%). Testing of an additional panel of the 19 most prevalent CFTR mutations among the Caucasian population in Europe, including G542X, N1303K, 1717-1G>T, W1282X, G551D, R553X, R1162X, R334W, R117H, 621+1G>T, 3849+10kbC>T, 3659delC, 1078delT, R347P, 1.3 Ca gluconate and 4 mM Ca gluconate was added to compensate for the chelating effects of gluconate. Bath solutions generally reported as peak responses of V<sub>te</sub> and basal V<sub>te</sub> were recorded continuously. Values for the V<sub>te</sub> were referred to the serosal side of the epithelium. Voltage deflections obtained under conditions without the mucosa present (ΔV<sub>te</sub>) were subtracted from those obtained in the presence of the tissues. R<sub>te</sub> was calculated according to Ohm’s law [R<sub>te</sub> = (ΔV<sub>te</sub> - ΔV<sub>eq</sub>)/ΔI<sub>eq</sub>]. The equivalent short-circuit current (I<sub>eq</sub>) was determined from V<sub>te</sub> and R<sub>te</sub>, i.e., I<sub>eq</sub> = V<sub>io</sub>/R<sub>te</sub>. Tissues were allowed to equilibrate for 30 min before basal bioelectric properties were taken.

Cell culture, RNA isolation, and RT-PCR. HT-29 and T-84 colonic carcinoma cells were grown in culture as described previously (18). In brief, cells were grown in Dulbecco’s modified Eagle’s medium with (in mM) 10 Na<sup>+</sup>-Hepes buffer, 4 l-glutamine (with 0.04 g/l penicillin), 0.09 streptomycin, and 100 newborn calf serum in 5% CO<sub>2</sub>. Total RNA was isolated from superficial biopsies of rectal mucosa and from the human colonic cell lines HT-29 and T-84 using RNeasy spin columns (Qiagen, Hilden, Germany), as described previously (22), and was reverse transcribed at 37°C for 1 h using random primer and RT (Superscript RT, Life Technologies). The size of the expected 543-bp fragment of PAR-2 was amplified by PCR using the sense primer 5'-GGTTTGTGGTGGGTTTGCC-3' and antisense primer 5'-CATCACCATAGGGCAGAGG-3' (94°C for 60 s, 35 cycles of 94°C for 1 min, 57°C for 30 s, 72°C for 60 s). PCR products were visualized by loading an 8-μl sample on a 0.9% agarose gel using a 123-bp marker as a standard. The PCR product was subcloned into pBluescript SK (−) vector and sequenced using Thermo Sequenase I (Pharmacia) and a 373A DNA sequencer (Applied Biosystem).

Compounds and statistics. Amiloride, bumetanide, indomethacin, cyclopiazonic acid (CPA) IBMX, fosfokolin, TTX, trypsin (bovine, 9,820 U/mg protein), trypsin inhibitor (type III-O chicken egg white; 1 mg will inhibit 1.1 mg trypsin with an activity of 10,000 BAEU U/mg protein), and thrombin (bovine, 56 U/mg protein) were all obtained from Sigma (Deisenhofen, Germany). PAR-2-AP (SLIGRL-NH<sub>2</sub>) corresponding to the tethered ligand of mouse PAR-2 and the reverse peptide (RP; LRGIL-NH<sub>2</sub>) were synthesized by solid-phase methods and purification by high-pressure liquid chromatography (Big Biotech, Freiburg, Germany). All used chemicals were of highest grade of purity available. From some individuals, transepithelial measurements were performed on more than one tissue sample. When multiple samples were studied by the same protocol, data were averaged to obtain a single mean value for each individual subject. Continuous bilateral bath perfusion allowed to perform consecutive measurements under different experimental conditions on the same tissue, and all experiments were performed in a paired fashion, in which each tissue served as its own internal control. Data for transepithelial measurements are shown as original recordings or as means ± SE and are generally reported as peak responses of V<sub>te</sub> and I<sub>te</sub> (n = number of subjects). Statistical analysis was performed using paired Student’s t-test. Data obtained from CF and non-CF tissues were compared by unpaired Student’s t-test. P values <0.05 were accepted to indicate statistical significance.

RESULTS

PAR-2 are expressed in native human colonic epithelium and are activated by trypsin. RNA was prepared from superficial biopsies of rectal mucosa and from the human colonic cell lines HT-29 and T-84, and RT-PCR was performed using primers specific to the sequence of human PAR-2. A 543-bp fragment was obtained, which was verified as a PAR-2 fragment by subsequent cloning and sequencing (Fig. 1). This result confirmed expression of PAR-2 for distal colon and rectum, which
cation absorption or anion secretion, subsequent experiments were performed in the presence of amiloride to inhibit electrogenic Na⁺ absorption. Amiloride (10 μM, luminal) significantly inhibited \( V_{te} \) and \( I_{eq} \) by 0.6 ± 0.1 mV and 26.2 ± 3.3 μA/cm² \((n = 30)\), respectively. In the presence of amiloride, trypsin was added to either the luminal or basolateral side of normal rectal biopsies. Basolateral perfusion with trypsin (1 μM) induced a transient increase in lumen-negative \( V_{te} \) (\( \Delta V_{te} = -2.3 ± 0.7 \) mV) and \( I_{eq} \) (\( \Delta I_{eq} = -112.6 ± 35.8 \) μA/cm²) and significantly decreased \( R_{te} \) by -1.1 ± 0.4 Ωcm² \((n = 6)\), whereas luminal trypsin had no effect on transepithelial ion transport (\( \Delta V_{te} = 0.0 ± 0.0 \) mV; \( \Delta I_{eq} = -0.7 ± 0.7 \) μA/cm²; \( n = 6 \); Fig. 2, A and B).

After basolateral trypsin was added, \( V_{te} \) and \( I_{eq} \) typically returned to control values within 3–5 min and the perfusion was switched back to control buffer. In some tissues, we observed a further reduction in \( V_{te} \) and \( I_{eq} \) below control values that was sustained for 10–30 min after trypsin washout. To address the possibility of trypsin-mediated inhibition of anion secretion in the late phase of the response, we performed experiments in which trypsin was added for 20 min. As shown in Fig. 2C, 1 μM trypsin induced an initial increase followed by sustained reductions of \( V_{te} \) (\( \Delta V_{te} = 0.9 ± 0.1 \) mV) and \( I_{eq} \) (\( \Delta I_{eq} = 40.3 ± 5.8 \) μA/cm²; \( n = 5 \)) below baseline values. To examine whether inhibition of anion secretion in the late phase of the response was concentration dependent, we performed similar experiments with lower concentrations of trypsin (10 and 100 nM; basolateral). Whereas 100 nM trypsin elicited qualitatively similar responses, we did not detect sustained inhibition of ion transport by 10 nM trypsin (Fig. 2C). According to these data, basolateral but not luminal trypsin induced transient activation and, at higher concentrations, sustained inhibition of anion secretion.

To test whether trypsin-activated receptors are localized on epithelial cells or on subepithelial neuronal fibers, we performed experiments in which neurotransmitter release from the enteric nervous system was blocked by TTX. Perfusion with TTX (1 μM, basolateral) for 20 min had no effect on basal \( V_{te} \) and \( I_{eq} \).

Table 1. Bioelectrical properties of rectal biopsies from normal individuals and CF subjects under basal conditions, after treatment with amiloride (10 μM, luminal), indomethacin (10 μM, basolateral), and after cAMP-dependent stimulation with IBMX (100 μM) and Fors (10 μM)

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Amiloride</th>
<th>Indomethacin</th>
<th>IBMX/Fors</th>
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<tr>
<td>Normal (30)</td>
<td></td>
<td></td>
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<tr>
<td>( V_{te} ), mV</td>
<td>-1.4 ± 0.2</td>
<td>-0.8 ± 0.1 atas</td>
<td>-0.2 ± 0.0†</td>
<td>-1.9 ± 0.2‡</td>
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<tr>
<td>( R_{te} ), Ωcm²</td>
<td>23.7 ± 2.0</td>
<td>28.8 ± 2.4</td>
<td>29.4 ± 2.2‡</td>
<td>25.1 ± 1.8‡</td>
</tr>
<tr>
<td>( I_{eq} ), μA/cm²</td>
<td>-58.2 ± 5.7</td>
<td>-32.1 ± 4.5</td>
<td>-5.3 ± 1.3‡</td>
<td>-76.8 ± 7.6‡</td>
</tr>
<tr>
<td>CF (11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( V_{te} ), mV</td>
<td>-1.8 ± 1.0</td>
<td>0.6 ± 0.1§</td>
<td>0.0 ± 0.1‡§</td>
<td>0.2 ± 0.1§</td>
</tr>
<tr>
<td>( R_{te} ), Ωcm²</td>
<td>43.3 ± 9.0</td>
<td>46.3 ± 9.7</td>
<td>46.9 ± 9.6</td>
<td>34.5 ± 6.0‡</td>
</tr>
<tr>
<td>( I_{eq} ), μA/cm²</td>
<td>-32.9 ± 11.3</td>
<td>12.8 ± 4.1‡</td>
<td>0.1 ± 2.2‡</td>
<td>6.8 ± 2.9§</td>
</tr>
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</table>

Values are means ± SE. *Statistical significance compared with basal properties; †statistical significance compared with amiloride; §statistical significance compared with indomethacin (paired t-test); ‡significant difference for the effects of amiloride, indomethacin, and IBMX/forskolin (Fors) compared with tissues from normal subjects. CF, cystic fibrosis; \( V_{te} \), transepithelial voltage; \( R_{te} \), transepithelial resistance; \( I_{eq} \), equivalent short-circuit current. Nos. in parentheses are nos. of subjects.
Importantly, TTX pretreatment had no effect on trypsin-mediated ion transport (trypsin peak response before TTX: $V_{te} = 0.7 \pm 0.3$ mV; $I_{eq} = -36.3 \pm 15.6$ $\mu$A/cm$^2$ vs. trypsin peak response after TTX: $V_{te} = -0.8 \pm 0.4$ mV; $I_{eq} = -36.4 \pm 12.6$ $\mu$A/cm$^2$; $n = 3$). These results indicate that PARs are localized on the basolateral membrane of colonocytes. As shown previously, basal and stimulated anion secretion in human colon can vary considerably after equilibration of tissues in Ussing chambers (25), most likely reflecting differences in the basal level of secretagogues released from individual native tissues. To control for the variability in endogenous autocrine or paracrine stimulation, the following experiments were performed in tissues prestimulated with IBMX (100 $\mu$M; basolateral) and forskolin (1 $\mu$M; basolateral). A concentration-response relationship was obtained for the effects of basolateral trypsin when added in concentrations ranging from 0.1 to 1 $\mu$M. $EC_{50}$ was 21 nM (Fig. 2, D and E). As shown in Fig. 2D, trypsin was able to activate short-circuit currents repeatedly in the same tissue when a recovery period of 20–30 min was allowed. These results point out to a highly specific action of trypsin on basolateral protease-activated receptors, probably PAR-2.

We obtained further evidence indicating that trypsin acts on PAR-2 by applying a PAR-2-specific AP (SLIGRL-NH$_2$). AP corresponds to the tethered ligand of cleaved PAR-2 and therefore does not require enzymatic cleavage for receptor activation. As summarized in Fig. 3, AP at concentrations as low as 10 $\mu$M elicited larger responses ($V_{te} = -0.4 \pm 0.1$ mV; $I_{eq} = -30.7 \pm 4.5$ $\mu$A/cm$^2$; $n = 4$), and luminal application of AP was without
effects on $I_{eq}$ (data not shown). In contrast, a peptide made from a reverse amino acid sequence (RP, LRGILS-NH$_2$) had no effect on transepithelial ion transport (Fig. 3). Therefore, these data suggest specific stimulation of electrolyte transport by activation of basolateral PAR-2.

**Cl$^-$ secretion is not activated by thrombin and is inhibited by trypsin inhibitor.** PAR activation requires specific enzymatic cleavage by trypsin or trypase, which releases a tethered ligand that activates its own receptor (12). It has been shown that trypsin-mediated activation of PAR-2 on epithelial cells can be abolished by preincubation of trypsin with trypsin inhibitor (26). To investigate whether the effects of trypsin on ion transport in human rectal biopsies could be antagonized by trypsin inhibitor, we compared the effects of trypsin in the absence and presence of trypsin inhibitor (1:1). After trypsin was pretreated with the inhibitor, the effects of trypsin on $I_{eq}$ were attenuated significantly from $-56.8 \pm 11.6\text{ to } -11.8 \pm 5.6\ \mu\text{A/cm}^2 (\Delta V_{te}$ was reduced from $-1.0 \pm 0.1\text{ to } -0.2 \pm 0.1\ \text{mV}; n = 4$; Fig. 4, A and B). Trypsin has been shown to act on several PARs in the rank order PAR-2 $\geq$ PAR-4 $>$ PAR-1 = PAR-3, whereas thrombin is a strong activator of PAR-1, PAR-3, and PAR-4 (12). To further characterize the PAR involved in trypsin-induced ion transport in the human distal colon, the effects of both trypsin and thrombin were compared. As depicted in Fig. 4, C and D, thrombin (1 $\mu$M, basolateral) failed to induce Cl$^-$ secretion, whereas trypsin activated lumen-negative responses of $V_{te}$ and $I_{eq}$ in paired experiments. Together, the present experiments strongly suggest that trypsin acts on basolateral PAR-2 in the human distal colon.

**Trypsin-induced Cl$^-$ secretion depends on cAMP-activation and requires luminal CFTR Cl$^-$ channels.** We previously demonstrated that Ca$^{2+}$-mediated Cl$^-$ secretion induced by cholinergic stimulation of human distal colon relies on functional CFTR as the luminal Cl$^-$ channel (24). However, previous results obtained on cultured cells from the kidney collecting duct and pancreatic duct suggest activation of Ca$^{2+}$-dependent Cl$^-$ channels by stimulation of PAR-2 (2, 26). Given these results, we asked whether trypsin may activate otherwise dormant non-CFTR Cl$^-$ channels in human rectal mucosa. To that end, we examined the effects of trypsin in the absence and presence of cAMP-dependent stimulation and compared the responses obtained in tissues from normal individuals and CF patients. The effects of trypsin were compared with the effects of carbachol (100 $\mu$M, basolateral) in a strictly paired fashion under three different conditions: 1) under basal conditions, 2) after perfusion with indomethacin, and 3) after cAMP-dependent stimulation with IBMX (100 $\mu$M) and forskolin (1 $\mu$M, both basolateral). As shown in Fig. 5A, trypsin-induced anion secretion required coactivation by cAMP. Under baseline conditions with variable CFTR activity, trypsin induced a lumen-negative secretory response of $-66.6 \pm 22.8\ \mu\text{A/cm}^2 (\Delta V_{te} = -1.3 \pm 0.5\ \text{mV}; n = 10)$. Treatment with the cyclooxygenase inhibitor indomethacin inhibits prostaglandin synthesis, including the formation of PGE, which has been identified as a major endogenous agonist of cAMP-dependent Cl$^-$ secretion in human colon.
Indomethacin (10 μM, basolateral, 60 min) pretreatment abolished trypsin-induced ion transport almost completely (ΔVte = -0.1 ± 0.1 mV; ΔIeq = -4.8 ± 2.2 μA/cm²; n = 10), whereas subsequent activation with IBMX and forskolin induced a sustained secretory response and resulted in a significant increase in trypsin-activated anion secretion (ΔVte = 1.4 ± 0.4 mV; ΔIeq = -99.8 ± 29.5 μA/cm²; n = 10) (Fig. 5, A and C). Ion-substitution experiments showed that cAMP and trypsin-induced secretory responses were carried by Cl⁻. In the presence of IBMX and forskolin, replacement of extracellular Cl⁻ by gluconate (bilateral equimolar replacement of 142 mM Cl⁻ by gluconate; 5 mM Cl⁻ remaining) resulted in a significant inhibition of Vte and Ieq (ΔVte = 1.5 ± 0.2 mV; ΔIeq = 46.9 ± 15.0 μA/cm²; n = 4). Furthermore, trypsin-mediated secretion was almost abolished in the presence of low Cl⁻ buffer (trypsin response under normal Cl⁻: ΔVte = -2.6 ± 0.3 mV; ΔIeq = -80.0 ± 23.8 μA/cm² vs. trypsin response under low Cl⁻: ΔVte = -0.2 ± 0.1 mV; ΔIeq = -2.2 ± 0.3 μA/cm²; n = 4), demonstrating that PAR-2 activation induces Cl⁻ secretion in normal human colon. In contrast, in tissues obtained from CF patients (n = 10), trypsin failed to induce Cl⁻ secretion under all experimental conditions. Instead, trypsin activated a lumen-positive K⁺ secretory response, which was +12.2 ± 4.5 μA/cm² (ΔVte = 0.4 ± 0.1 mV; n = 10) in the presence of indomethacin. These experiments demonstrate that trypsin-induced Cl⁻ secretion depends on the presence of functional CFTR as the luminal Cl⁻ channel. Although the magnitude of cholinergic secretion in both normal and CF tissues was significantly larger compared with trypsin-induced secretion, both agonists demonstrated a very similar behavior. This observation may suggest that both agonists share a common intracellular signal-transduction pathway.

Trypsin-induced Cl⁻ secretion requires basolateral Cl⁻ uptake and activation of K⁺ channels. In the presence of amiloride, lumen-negative responses of Vte and Ieq are caused by anion secretion. Due to the lack of highly specific inhibitors of CFTR Cl⁻ channels, we used bumetanide (100 μM, basolateral), an inhibitor of
the Na\(^+\)-K\(^+\)-2 Cl\(^-\) cotransporter, to block basolateral Cl\(^-\) uptake. As shown in Fig. 6, both cAMP-activated and trypsin-induced anion secretion was almost completely abolished by bumetanide, indicating that trypsin activated transepithelial Cl\(^-\) secretion.

We have shown previously that cholinergic stimulation of Cl\(^-\) secretion depends on basolateral Ca\(^{2+}\)-activated K\(^+\) channels (24). Here we examined the effect of K\(^+\)-channel inhibitors (BaCl\(_2\) and clotrimazole) on trypsin-induced secretion in human distal colon. BaCl\(_2\) (5 mM, basolateral) significantly inhibited the sustained Cl\(^-\) secretory response in the presence of cAMP stimulation (IBMX/forskolin). Furthermore, the trypsin (1 mM, basolateral)-induced Cl\(^-\) secretory response was significantly reduced from \(-148.5 \pm 4.3\) to \(-62.1 \pm 2.7\) μA/cm\(^2\) (ΔV\(_{\text{te}}\) was reduced from \(-2.5 \pm 0.6\) to \(-1.2 \pm 0.4\) mV; \(n = 7\)) (Fig. 6, C and D). Similar observations were made using clotrimazole as a more specific inhibitor of Ca\(^{2+}\)-dependent K\(^+\) channels. In the presence of IBMX and forskolin, clotrimazole (30 μM) inhibited I\(_{\text{eq}}\) from \(-26.7 \pm 8.2\) to \(-9.2 \pm 2.5\) μA/cm\(^2\) and abolished the trypsin-induced response almost completely from \(-100.5 \pm 37.4\) to \(-3.9 \pm 2.5\) μA/cm\(^2\) (ΔV\(_{\text{te}}\) was reduced from \(-1.7 \pm 0.6\) to \(-0.5 \pm 0.2\) mV; \(n = 3\)). These results indicate that trypsin-induced Cl\(^-\) secretion requires coactivation of basolateral K\(^+\) channels.

PAR-mediated ion transport requires an increase in intracellular Ca\(^{2+}\). Activation of PAR-2 by trypsin has been shown to increase intracellular Ca\(^{2+}\) and generation of PGE\(_2\) (12). The present results suggested that ion transport activated by stimulation of PAR-2 is

Fig. 5. Effects of Tryp (1 μM, bl) and carbachol (CCH) (100 μM, bl) on ion transport under basal conditions and in the absence (indomethacin, 10 μM, bl) and presence (IBMX, 100 μM and Fors 1 μM, bl) of cAMP-dependent activation of rectal biopsies from normal and CF subjects. All experiments were performed in the presence of amiloride (10 μM, luminal). A: in normal tissues (non-CF), Tryp and CCH induced a transient Cl\(^-\) secretory response under basal conditions that was further increased after cAMP stimulation of Cl\(^-\) secretion. In the presence of indomethacin (60 min), Cl\(^-\) secretion was abolished and CCH induced a reversed lumen-positive K\(^+\) secretory response. A: in CF tissues, Tryp- and CCH-induced Cl\(^-\) secretion was defective, and both agonists activated transient K\(^+\) secretion, which did not depend on cAMP activation. Time gaps between recordings were 40 min. Note that the magnification was changed during the course of the original recordings. C: summary of Tryp- and CCH-induced I\(_{\text{eq}}\) obtained from experiments as shown in A (non-CF) and B (CF). Mean values ± SE. *Statistical significance for the effects of Tryp and CCH. #Significant difference for the effects of Tryp vs. CCH, **significant difference for Tryp- and CCH-induced I\(_{\text{eq}}\) compared with basal conditions, $Significant difference for Tryp- and CCH-induced I\(_{\text{eq}}\) compared with indomethacin (paired t-test). §Significant difference compared with non-CF (unpaired t-test).
caused by increase in intracellular Ca\textsuperscript{2+}. To further confirm the role of Ca\textsuperscript{2+} as the mediator of PAR-2 effects in human colon, tissues were treated for 20 min with the Ca\textsuperscript{2+}-ATPase inhibitor CPA (50 \mu M, both sides). Inhibition of ATPase-dependent Ca\textsuperscript{2+} reuptake into endoplasmic stores is expected to cause a transient increase in intracellular Ca\textsuperscript{2+}, followed by inhibition of agonist-induced store release. Accordingly, addition of CPA induced an initial Cl\textsuperscript{-} secretory response of \(-62.8 \pm 13.9\) \mu A/cm\textsuperscript{2} (\(\Delta V_{te} = -0.6 \pm 0.1\) mV; \(n = 5\)). After 20 min of CPA treatment, a stable plateau was reached, which was not different from precontrol values. As shown in Fig. 7, A and B, the effect of trypsin was almost completely abolished in the presence of CPA. Similar to the trypsin response, cholinergic stimulation by carbachol (CCH) was also inhibited in the presence of CPA (Fig. 7, C and B). To address the role of extracellular Ca\textsuperscript{2+} in PAR-2-mediated secretion, we compared the effect of trypsin in low (1 \mu M) and normal Ca\textsuperscript{2+} (1.3 mM) bath solutions. Bilateral perfusion with 1 \mu M Ca\textsuperscript{2+} had no effect on \(V_{te}\) or \(I_{eq}\). In the presence of low-Ca\textsuperscript{2+} buffer, the effect of trypsin was almost abolished (trypsin response under normal Ca\textsuperscript{2+}: \(\Delta V_{te} = -1.2 \pm 0.2\) mV; \(\Delta I_{eq} = -56.5 \pm 7.9\) \mu A/cm\textsuperscript{2} vs. trypsin response under low Ca\textsuperscript{2+}: \(\Delta V_{te} = -0.3 \pm 0.1\) mV; \(\Delta I_{eq} = -23.2 \pm 11.8\) \mu A/cm\textsuperscript{2}; \(n = 3\)). Similar observations were obtained when tissues were stimulated with CCH in normal compared with low Ca\textsuperscript{2+} solution (data not shown). These data suggest that activation of PAR by trypsin and stimulation of Cl\textsuperscript{-}
secretion in the human colon depends on intracellular Ca\(^{2+}\) signaling by endoplasmic stores release and extracellular Ca\(^{2+}\) entry.

**DISCUSSION**

Trypsin can act as a signal molecule that specifically regulates cells by cleaving and activating PAR-2 (12). Trypsin has previously been shown to regulate ion transport in cultured epithelial cells from the pancreatic duct and kidney collecting duct by acting on PAR-2 located on the basolateral side of the epithelium (2, 26). Whereas a physiological role of PAR-2 detected in cultured kidney cells remains obscure, a protective function during pancreatitis was claimed for PAR-2 expressed on the basolateral side of pancreatic duct cells. A recent report elucidated the role of PAR-2 in regulating salivary and pancreatic exocrine secretion in vivo in rats and mice (20). It was found that stimulation of PAR-2 transiently increased Cl\(^{-}\) secretion, which was followed by a sustained inhibition of amiloride-sensitive short-circuit currents (11). It is therefore likely that PAR-2 play a role in regulation of Na\(^{+}\) absorption and Cl\(^{-}\) secretion in human airways.

A basolateral PAR has been implicated in trypsin-mediated activation of ion transport in rat jejunum (33). From differences in the potency profile of the originally described AP (SLIGRL-NH\(_2\)) and an alternative PAR-2-selective AP (LIGRLO-NH\(_2\)), the authors suggested that a receptor different from PAR-2 is activated by trypsin in rat intestine. However, such a...
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receptor has not yet been identified. It cannot be ruled out that 1) different PAR-2 subtypes exist that demonstrate different potency profiles for these synthetic agonists or that 2) the binding affinity of PAR-2 for different peptides is modulated by coexpression of other associated membrane proteins in the native epithelium. PAR-2 expression was detected in both basolateral and luminal membranes of enterocytes of the rat jejunum in another study (21). Stimulation of cultured enterocytes by basolaterally applied trypsin or PAR-2-activating peptide induced a release of arachidonic acid, generation of inositol 1,4,5-trisphosphate, and production and secretion of PGE2 (21). Thus pancreatic trypsin in concentrations usually present in the lumen of the jejunum may be able to activate PAR-2 located in either luminal or basolateral membranes of enterocytes (14). The data shown in the present study demonstrate that basolateral PAR-2 are responsible for trypsin-mediated activation of Cl\(^-\) secretion in human distal colon. Because trypsin had no effect on ion transport when applied from the luminal side, the receptors mediating trypsin-activated ion secretion are strictly located on the basolateral side of the epithelium. Differences in luminal and basolateral PAR-2 expression in small intestine and distal colon may indicate different physiological functions but could also be due to species differences. Importantly, pretreatment of tissues with TTX had no effect on trypsin-mediated Cl\(^-\) secretory responses, indicating that PAR-2 is expressed on the basolateral membrane of epithelial cells rather than on subepithelial structures, e.g., neuronal fibers. To assess trypsin-mediated Cl\(^-\) secretion, experiments were generally performed in the presence of amiloride to inhibit electrogenic Na\(^+\) absorption. However, we made quantitatively similar observations when trypsin was added under basal conditions in the absence of amiloride. Although it cannot be excluded that the increase in \(V_{te}\) and \(I_{se}\) observed in the absence of amiloride is related to activation of cation absorption, the lumen-negative trypsin response in the absence of amiloride would also be in agreement with anion secretion under physiological conditions. This is well conceivable, because colonic crypts are composed of functionally distinct compartments, with Cl\(^-\) secretion predominantly taking place at the crypt basis and Na\(^+\) absorption at the surface epithelium (15). Experiments using trypsin concentrations \(\geq 100 \, \text{nM}\) indicate that PAR-2 activation may actually inhibit anion secretion in the continuous presence of the agonist. Alternatively, the transient decrease in \(V_{te}\) and \(I_{se}\) may be due to activation of transepithelial K\(^+\) secretion. Furthermore, this observation could be caused by more complex mechanisms; e.g., endogenous PAR-2 activation in native tissues could contribute to basal and stimulated ion transport, and addition of trypsin at high concentrations may lead to inactivation of these PAR-2 by cleavage and/or internalization. However, the trypsin concentrations (\(\geq 100 \, \text{nM}\)) required to induce inhibition of \(V_{te}\) and \(I_{se}\) in the plateau phase of the trypsin response were much higher than the EC\(_{50}\) (21 nM). Therefore, inhibition of ion transport by PAR-2 appears unlikely under physiological conditions.

Previous studies on cultured renal and pancreatic cells suggested activation of luminal Cl\(^-\) and basolateral K\(^+\) channels by trypsin along with an increase in intracellular Ca\(^{2+}\) (2, 26). From the present experiments on human native colonic mucosa, there is significant evidence for activation of basolateral Ca\(^{2+}\)-dependent K\(^+\) channels. However, stimulation of PAR-2 did not activate CFTR or alternative Ca\(^{2+}\)-dependent Cl\(^-\) channels in distal colonic tissues. This result confirms previous studies demonstrating the absence of Ca\(^{2+}\)-activated Cl\(^-\) channels in human and mouse distal colon (16, 24, 25). Cholinergic stimulation of Cl\(^-\) secretion in the mammalian colon has been studied in great detail (6, 17, 24, 25). Here, we compared the effects of carbachol and trypsin on normal and CF rectal biopsies and found qualitatively identical responses for both agonists, suggesting that CCH and trypsin activate similar ion conductances. Moreover, depleting the endoplasmic reticulum from Ca\(^{2+}\) renders the tissue unresponsive to trypsin. Thus trypsin is likely to act via release of IP\(_3\), an increase in intracellular Ca\(^{2+}\), and activation of basolateral Ca\(^{2+}\)-dependent K\(^+\) channels, which enhances the driving force for luminal Cl\(^-\) secretion.

An important aspect of the present results is the reversibility of the PAR-2 mediated effects. Trypsin cleaves PAR-2 and activates the receptor irreversibly. Resensitization is due to mobilization of large Golgi stores and synthesis of new receptors (4). A very rapid partial recovery was observed in the present study already after 15 min, which is similar to what has been observed in the airways (7). This finding indicates a very rapid turnover of PAR-2 in native tissue and supports their significance in the regulation of ion transport in vivo. PAR-2 is also activated by inflammatory mediators such as trypptide, which is released during mast cell degranulation. In the human gut, mast cells are resident in the mucosa associated lymphatic tissue, where they secrete other proinflammatory cytokines, including tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) (3). TNF-\(\alpha\) and interleukin-1 as well as bacterial lipopolysaccharides have been shown to induce a sustained 10-fold increase in PAR-2 expression in endothelial cells (28), supporting participation of PAR-2 in the inflammatory response observed in chronic inflammatory bowel disease. Interestingly, TNF-\(\alpha\) has been shown to be an essential mediator in inflammatory bowel disease, and recent clinical trials have shown that treatment with TNF antibody downregulates inflammation successfully in patients with Crohn’s disease who did not respond to conventional treatment (1, 31). Inflammatory bowel disease, as it occurs in Crohn’s disease, is characterized by mast cell infiltration, which forms an essential component of intestinal granuloma (23, 29). Moreover, mast cells have been implicated in affecting ion transport in the human intestine in a previous study, and changes in ion transport have been found in patients with inflammatory bowel disease (9). These results are in parallel

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with those showing activation of colonic myocytes by mast cell tryptase and consecutive disturbances in colonic motility in Crohn’s disease (8). Together with the results from this study demonstrating PAR-2-mediated activation of ion transport in human distal colon, epithelial PAR-2 may contribute to the pathophysiology of inflammatory bowel disease and thus may form a novel pharmacological target.

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