AGONISTS OF PROTEASE-ACTIVATED RECEPTORS 1 AND 2 STIMULATE ELECTROLYTE SECRETION FROM MOUSE GALLBLADDER

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\textbf{Running Head:} PARs localization and secretory function in gallbladder

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Cholecystitis is one of the most common gastrointestinal diseases. Inflammation induces the activation of proteases that can signal to cells by cleaving protease-activated receptors (PARs) to induce hemostasis, inflammation, pain and repair. However, the distribution of PARs in the gallbladder is unknown, and their effects on gallbladder function have not been fully investigated. We localized immunoreactive PAR$_1$ and PAR$_2$ to the epithelium, muscle and serosa of mouse gallbladder. mRNA transcripts corresponding to PAR$_1$ and PAR$_2$, but not PAR$_4$, were detected by RT-PCR and sequencing. Addition of thrombin and PAR$_1$-selective activating peptide (AP, TFLLRN-NH$_2$) to the serosal surface of mouse gallbladder mounted in an Ussing chamber stimulated an increase in short circuit current ($I_{sc}$) in wild-type but not PAR$_1^{-/-}$ mice. Similarly, serosally-applied trypsin and PAR$_2$-AP (SLIGRL-NH$_2$) increased $I_{sc}$ in wild-type but not PAR$_2^{-/-}$ mice. Proteases and APs strongly inhibited electrogenic responses to subsequent stimulation with the same agonist, indicating homologous desensitization. Removal of HCO$_3^-$ ions from the serosal buffer reduced responses to thrombin and trypsin by $>80\%$. Agonists of PAR$_1$ and PAR$_2$ increase [Ca$^{2+}$], in isolated and cultured gallbladder epithelial cells (GBEC). Cyclooxygenase (COX)-2 inhibitor, meloxicam and an inhibitor of cystic fibrosis transmembrane conductance regulator (CFTR) prevented the stimulatory effect of PAR$_1$ but not PAR$_2$. Thus, PAR$_1$ and PAR$_2$ are expressed in the epithelium of the mouse gallbladder, and serosally applied
proteases cause a HCO₃⁻ secretion. The effects of PAR₁ but not PAR₂ depend on generation of prostaglandins and activation of CFTR. These mechanisms may markedly influence fluid and electrolyte secretion of the inflamed gallbladder, when multiple proteases are generated.

**Key words:** PARs, bicarbonate transport, receptor, epithelial transport, gallbladder.
INTRODUCTION

Inflammation of the gallbladder (cholecystitis) is one of the most common and clinically important digestive diseases. Although cholecystitis is usually associated with gallstones, it also occurs in their absence. The many complications from biliary disease range from simple biliary colic to ascending cholangitis, septic shock and death. Despite its prevalence, the molecular mechanisms of cholecystitis are poorly understood. No specific therapy for biliary inflammation currently exists. Treatments consist of antibiotics, invasive biliary decompression or surgical removal of the inflamed gallbladder.

Inflammation of many tissues results in the activation and secretion of serine proteases from the circulation, inflammatory cells and epithelial tissues, which can participate in the inflammatory response (32; 39). Trypsin has been documented in the bile of patients with cholecystitis (47), and digestive proteases may be present in the gallbladder under normal conditions when a closed sphincter of Oddi shunts pancreatic trypsin into the biliary tract (1; 46; 48). However, the effects of these proteases on gallbladder function have not been fully studied.

Certain serine proteases signal to cells by cleaving protease-activated receptors (PARs), a family of four G-protein-coupled receptors that are activated by proteolytic cleavage (32; 39). Thrombin activates PAR₁, PAR₃ and PAR₄ (24; 26; 49), whereas pancreatic trypsin, coagulation factors VIIa and Xa, mast cell tryptase and neutrophil proteases all activate PAR₂ (6; 10; 11;
Kallikreins are able to activate PAR 1, 2 and 4 (38). Proteases cleave within the extracellular N-terminal domains of PARs to expose tethered ligand domains, which bind to and activate the cleaved receptors (37; 49). Synthetic peptides corresponding to the tethered ligands (activating peptides, APs) can trigger PARs by directly binding to the receptors, making them useful reagents to investigate the functions of these receptors. PARs are widely distributed and participate in multiple processes (32; 39). The proteases that activate PARs are often generated or secreted during injury and inflammation, and PARs mediate responses to these stimuli, including hemostasis, inflammation, pain and tissue repair. The role of PARs in the gallbladder remains to be defined.

We sought to determine the expression and possible functions of PARs in the gallbladder epithelium. PARs are highly expressed by epithelial cells of the intestine, pancreatic duct and airway, where prominent effects of agonists are to regulate fluid and electrolyte secretion and paracellular permeability. Agonists of PAR$_1$ stimulate chloride secretion in the intestinal epithelium (8), and agonists of PAR$_2$ also regulate the epithelial cells of the digestive tract to stimulate generation of prostaglandins in the intestine (27), promote secretion of chloride ions in the intestine and pancreatic duct (17; 36), and increase paracellular permeability of colonocytes to macromolecules and bacteria (12; 25). However, which PARs are expressed by
gallbladder epithelium is unknown, as are the effects of proteases and selective PAR agonists on electrolyte secretion, a major function of the gallbladder.

Given the prominent expression of PARs in epithelial cells of the gastrointestinal tract, airway and kidney, and the effects of agonists on electrolyte secretion in these tissues (19; 20), we hypothesized that PARs are highly expressed by gallbladder epithelial cells where they control ion transport. Our aims were to: (1) examine the expression and localization of PARs in the mouse gallbladder using immunohistochemistry and RT-PCR; (2) investigate the effects of proteases and selective PAR agonists on electrolyte secretion by the gallbladder mounted in an Ussing chamber; (3) identify the receptors responsible for these effects by using tissues from PAR-deficient mice; and (4) determine the mechanisms by which PAR agonists affect electrolyte secretion by using pharmacological antagonists.

MATERIALS AND METHODS

Reagents. Trypsin (bovine pancreas), thrombin (bovine plasma), forskolin, amiloride, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS), 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), genistein, glybenclamide, bumetanide, furosemide, tetrodotoxin, indomethacin, ketorolac and meloxicam were from Sigma-Aldrich (St. Louis, MO, USA).
APs corresponding to the tethered ligand of mouse PAR₁ (SFLLRN-NH₂), *Xenopus* PAR₁ (TFLLRN-NH₂) and mouse PAR₂ (SLIGRL-NH₂), and their respective reverse sequences, NRLIFS-NH₂, NRLLFT-NH₂ and LRGILS-NH₂, which were used as inactive controls, were from Genemed Synthesis (South San Francisco, CA, USA). TFLLRN-NH₂ is highly selective for PAR₁, whereas SFLLRN-NH₂ is not (22). Thiazolidinone (2-thioxo-4-thiazolidinone) CFTR inhibitor-172 (CFTRinh-172) was a generous gift from Dr. A. S. Verkman, (University of California, San Francisco). Other reagents were from Fisher Scientific (Fairlawn, NJ, USA).

Gene Accession numbers: PAR₁: NM 010169; PAR₂: NM 007974.

**Mice.** Male CD-1 mice (4-6 months, 35-45 g) were from Charles River (Willington, MA, USA) and were used for all experiments unless stated otherwise. PAR₁ and PAR₂ deficient mice were from Dr. S.R. Coughlin (University of California, San Francisco) (15; 30). Heterozygous PAR₁- and PAR₂- deficient mice were bred and the litters were genotyped for identification of homozygote mice. The mice were fed standard laboratory chow and given water *ad libitum*. Mice were killed using sodium pentobarbital (200 mg/kg ip) and bilateral thoracotomy. The Institutional Animal Care and Use Committee approved all procedures.

**RT-PCR analysis of PAR₁, PAR₂ and PAR₄.** Freshly excised gallbladders were immediately placed into liquid nitrogen and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA). Genomic DNA was eliminated by using DNase I (Ambion Inc., Austin, TX, USA). RNA
(1.0 μg) was reverse transcribed using the AMV-RT system (Promega, Madison, WI, USA), and cDNA was amplified with Taq polymerase (Invitrogen). Primers to mouse PAR1 (forward, 5’CCTATGAGCCAGCCAGAATC-3’; reverse, 5’ACGTTCACAGGAAGGCTGAC-3’) were chosen to amplify a 305 bp fragment. Primers to mouse PAR2 (forward, 5’-TGGGAGGTATCACCCTCTTG-3’; reverse, 5’-CCAGGTG GCCCATGTAAATC-3’) were chosen to amplify a 330 bp fragment. Primers to mouse PAR4 (forward 5’-TGCTGTATCCTTTGGTGC-3’; reverse 5’-CATGAGCAGAATGGTGATG-3’) were chosen to amplify an expected product size of 371 bp. The PCR conditions were: denaturation for 10 min at 94°C, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, followed by a final elongation at 72°C for 10 min. To exclude contamination by genomic DNA, control RT-PCR reactions omitted reverse transcriptase AMV-RT for each sample. PCR products were analyzed by electrophoresis on a 2% agarose gel with ethidium bromide, and were identified by sequencing.

Immunohistochemistry. Gallbladders were removed and placed into 4% paraformaldehyde, 100 mM PBS (pH 7.4) at 4°C overnight. Tissues were transferred to 30% sucrose in PBS for 24 hours at 4°C. Gallbladders were embedded in Optical Cutting Temperature compound and frozen sections (8 μm) were prepared. Tissue sections were rehydrated in PBS (pH 7.4) for 15 min, and incubated in blocking buffer using the Vectastain ABC kit (Vector Laboratories,
Sections were incubated with antibodies to PAR₁ (sc-8204, Santa Cruz Biotechnology, Santa Cruz, CA) (1:500 dilution) or PAR₂ (sc-8207, Santa Cruz Biotechnology (1:750 dilution) overnight at 4°C. After three rinses in PBS, sections were incubated with the secondary antibodies. Vectastain ABC and DAB peroxidase substrate kits (Vector Laboratories) were used for detection according to the manufacturer's instructions. For controls, PAR₁ and PAR₂ antibodies were pre-absorbed with the receptor fragment used for immunization (10 µM, 72 h, 4°C).

**Isolation and culture of gallbladder epithelial cells.** Gallbladder epithelial cells (GBEC) were isolated using a previously described procedure (29). Briefly, gallbladders (minimum of five) are dissected free of connective tissue and placed in ice cold culture media. They are opened, rinsed with cold PBS, and placed in pre-warmed (37°C) trypsin + EDTA, PBS for 45 min. The released epithelial cells were collected, filtered, and washed with Minimal Essential Media +10% fetal calf serum (EMEM). Cells were centrifuged, resuspended in insulin-transferin-selenium supplemented EMEM, and plated onto 15 mm, circular, collagen-coated glass cover-slips for measurement of intracellular calcium mobilization, [Ca²⁺], and for cytokeratin staining (Santa Cruz Biotechnology).

**Measurement of [Ca²⁺].** GBEC were isolated and cultured for 7-14 days. GBEC were incubated in PBS containing 0.1% BSA, 7µM Fura-2/AM and 0.2% pluronic for 20 min at 37°C.
Cells were washed in PBS+ 0.1% BSA, placed into a temperature-controlled 0.5 ml incubation chamber mounted on the stage of a Zeiss Axiovert microscope, and observed with a Zeiss Fluar x 40 objective (numerical aperture 1.3). Agonists were added directly to the chamber from concentrated stock solutions. Images were obtained using an integrating CCD camera (Stanford Photonics, Stanford, CA) and a video microscopy acquisition program (Axon Imaging Workbench 5.6, Axon Industries, Foster City, CA). Changes in fluorescence were measured at 340 nm and 380 nm excitation and 510 nm emission. The ratio of the fluorescence intensity at 340 and 380 nm, which is proportional to the $\left[\text{Ca}^{2+}\right]_i$ was calculated. Fresh GBEC were studied with each agonist concentration, and a minimum of 15 epithelial cells were analyzed on 3 experiments on different days.

**Transepithelial electrical measurements.** Gallbladders were removed under a dissecting microscope, immediately placed in ice-cold Krebs solution (KBS), and opened longitudinally to expose the luminal surface. They were mounted onto small caliber sliders (surface area of 0.031 cm$^2$) and transferred to Ussing-style chambers for continuous measurement of short-circuit current ($I_{sc}$) using a VCC MC6 clamp system (EasyMount Diffusion Chambers-Physiological Instruments, San Diego, CA, USA). Electrode agar bridges were made with 3-4% agarose in 3M KCl saturated with Ag and AgCl$_2$. The $I_{sc}$ was determined by clamping trans-epithelial voltage ($V_{te}$) to 0 mV using the voltage clamp mode of the amplifier. An increase in $I_{sc}$
may be due to either electrogenic cation absorption or anion secretion, or some combination of both. Tissues were bathed in KBS containing 120 mM NaCl, 25 mM NaHCO₃, 3.3 mM KH₂PO₄, 0.8 mM K₂HPO₄, 1.2 mM MgCl₂, 1.2 mM CaCl₂ and 10 mM glucose at 37°C, pH 7.3, and bubbled with 95% O₂ -5% CO₂. Data were acquired using the Biopac Data Acquisition System (Santa Barbara, CA, USA). The optimal working concentrations of agonists were determined by generating dose-response curves. Agonists were added either to the serosal, luminal, or both surfaces of the gallbladder at the following concentrations: synthetic peptides, 100 µM; trypsin, 1 µM; and thrombin, 100 U/ml. Proteases were heat-inactivated by boiling for 5 min immediately before use. The time interval between successive agonist doses was 10 min with or without intervening washes. A change in short-circuit current (ΔIsc) was determined by subtracting the mean peak response after addition of the reagent from the baseline Isc. All Isc values are reported as an increase over baseline unless stated otherwise. Bicarbonate-free solutions were made by substituting NaHCO₃ and KH₂PO₄ with sucrose and adding 10 mM HEPES buffer. Chloride-free solutions were made by substituting NaCl with Na-glucuronate.

**Statistical analysis.** Data are presented as mean (S.D.) Tissues from at least three mice were used in all experiments. Comparisons between a single control and treatment mean were made using the unpaired, two-tailed Student t test. Comparisons between multiple treatment means
were made by ANOVA and a Dunn’s test. The limit of statistical significance was set at $P < 0.05$.

**RESULTS**

**Expression and localization of PAR₁ and PAR₂ in gallbladder.** To determine whether the gallbladder expressed PARs, we attempted to amplify PAR₁, PAR₂ and PAR₄ mRNA from whole gallbladder tissue by RT-PCR. Products of anticipated size were amplified for PAR₁ (305 bp) and PAR₂ (330 bp) (Fig. 1A). No product was amplified for the predicted 371 bp PCR fragment specific for PAR₄ mRNA in gallbladder, but was detected in mouse myocardium used as a positive control (Fig. 1B) (42). The purified PCR products were sequenced to confirm their identity. The sequenced PCR product from the lower second band from myocardium corresponded to the sequence of Mus musculus MYST histone acetyltransferase monocytic leukemia 4.

To determine the sites of PAR₁ and PAR₂ expression, we used immunohistochemistry and light microscopy. Immunoreactive PAR₁ was localized to epithelial cells of the gallbladder mucosa (Fig. 2A, upper panel). Prominent staining was also observed in the submucosal, muscular and serosal layers. Immunoreactive PAR₂ was localized mainly to the epithelial layer with weaker staining of the muscular and serosal layers (Fig. 2B, upper panel). Staining by both antibodies was abolished by preabsorption with the receptor fragments used for immunization,
confirming specificity (Fig. 2, lower panels). The prominent expression of PARs in the
gallbladder epithelium suggested a role of these receptors in regulating ion transport, which was
investigated in subsequent experiments.

**PAR1 and PAR2 agonists stimulate I<sub>sc</sub>.** To determine the role of PAR<sub>1</sub> and PAR<sub>2</sub> in regulating
electrolyte secretion and to confirm functional expression of PARs, we measured changes in I<sub>sc</sub>
to graded concentrations of agonists in whole gallbladder tissues. The baseline I<sub>sc</sub> was 17.4
µA/cm<sup>2</sup> (SD 28.5) in non-stimulated tissues (n=40 mice). When applied to the serosal
(basolateral) surface, thrombin (100 U/ml) stimulated a rapid increase in I<sub>sc</sub> of 52.2 µA/cm<sup>2</sup> (SD
30.6) over baseline (n=8) that returned to pre-stimulated levels by 5 min (Fig. 3A, upper panel).
Luminal (apical) treatment with thrombin stimulated smaller increases in I<sub>sc</sub> (35.4 µA/cm<sup>2</sup>; SD
23.8; n=4) that were slow in onset and sustained (Fig. 3A, lower panel). Heat-inactivated
thrombin had no effect (Fig. 3A, middle panel). The peptide agonist corresponding to the
tethered ligand of human PAR<sub>1</sub>, SFLLRN-NH<sub>2</sub> (100 µM) also increased I<sub>sc</sub> by 54.0 µA/cm<sup>2</sup> (SD
48.2; n=12), but only when applied to the serosal surface (not shown). Since SFLLRN-NH<sub>2</sub> also
activates PAR<sub>2</sub> (4) we assayed the PAR<sub>1</sub> selective analogue TFLLRN-NH<sub>2</sub>. Serosal stimulation
with TFLLRN-NH<sub>2</sub> (100 µM) caused a rapid increase in I<sub>sc</sub> of 71.2 µA/cm<sup>2</sup> (SD 31.1; n=7) (Fig.
3B, upper panel) with a similar efficacy but reduced potency to thrombin (EC<sub>50</sub> ~ 50 µM, Fig.
5A). However, TFLLRN-NH<sub>2</sub> had no effect when applied to the luminal surface (Fig. 3B, lower
The corresponding reverse sequences of the tethered ligand peptides RNLLFT-NH₂ (Fig. 3B, middle panel) and RNLLSF-NH₂ (not shown), which do not activate PAR₁, had no effect when applied serosally, confirming specificity.

We similarly examined the effects of PAR₂ agonists on electrogenic current. Trypsin (1 µM) caused a prompt increase in Iₛₑ when added to either serosal or luminal surfaces: 73.3 ± 29.4 µA/cm² (SD 29.4; n=6) and 60.1 ± 19.5 µA/cm² (SD 19.5; n=9), respectively (Fig. 4A, upper and lower panels). PAR₂-specific AP, SLIGRL-NH₂ (100 µM), increased Iₛₑ by 40.9 ± 22.1 µA/cm² (SD 22.1; n=10) when applied to the serosal surface with a reduced efficacy and potency relative to trypsin (EC₅₀ ~ 50 µM). SLIGRL-NH₂ had no effect when applied to the luminal surface. Both heat-inactivated trypsin and the reverse peptide sequence LRGILS-NH₂ had no effect on secretory current when added to either side, confirming specificity (Fig. 4A, B, middle panels).

Together, these results show that agonists of PAR₁ and PAR₂ increase Iₛₑ, especially when applied to the serosal surface. Therefore, functional expression of PAR₁ and PAR₂ is greater on the serosal surface than on the luminal surface. Accordingly, PAR agonists were added only to the serosal surface in subsequent experiments.

The Iₛₑ response to PAR activation undergoes desensitization. We examined desensitization of electrogenic responses to thrombin, trypsin and APs to verify activation of their corresponding receptors, PAR₁ and PAR₂. When thrombin was used as the first agonist,
the electrogenic response to TFLLRN-NH₂ was strongly desensitized and the response to a second challenge with thrombin (without an intervening wash) was abolished (Fig. 5A, upper panels). When TFLLRN-NH₂, was used as the first agonist, the response to a second equimolar challenge with TFLLRN-NH₂ was desensitized by 53% (SD 12; n=4) relative to the initial Iₛₑ response. TFLLRN-NH₂ also desensitized tissues to a second challenge with thrombin by 71% (SD 22; n=5) (Fig. 5A, lower panels). Together, these results suggest that thrombin and the PAR₁ AP activate the same receptor on the gallbladder serosal surface. Similarly, trypsin strongly desensitized the tissue to a second challenge with SLIGRL-NH₂ or trypsin (Fig. 5B, upper panels). SLIGRL-NH₂ desensitized tissues to a second challenge with SLIGRL-NH₂ by 55% (SD 16). However, SLIGRL-NH₂ did not desensitize the Iₛₑ response to a second challenge with trypsin (Fig. 5B, lower panel), perhaps a result of trypsin activation of PAR₁ (7; 45; 49). Overall, these findings suggest that trypsin induces transepithelial ion conductance by cleaving and activating PAR₂ in gallbladder tissue.

**Thrombin and trypsin stimulate Iₛₑ by activating PAR₁ and PAR₂, respectively.** Receptor desensitization by selective agonists suggested that proteases affect electrolyte secretion by activating PAR₁ and PAR₂. However, since proteases can have many actions, we studied their effects on Iₛₑ in PAR-deficient mice to confirm the mechanisms of protease-stimulated electrolyte secretion. Since both PAR₁ and PAR₂ knockout mice were bred to a C57BL6
background, we first evaluated the wild-type mice for functional expression of PAR\textsubscript{1} and PAR\textsubscript{2} as a necessary control. In wild-type C57B/6 mice, PAR\textsubscript{1} and PAR\textsubscript{2} agonists stimulated normal $I_{sc}$ responses (PAR\textsubscript{1} AP, TFLLRN-NH\textsubscript{2}, 51$\mu$A/cm$^2$; SD 38); PAR\textsubscript{2} AP, SLIGRL-NH\textsubscript{2}, 51$\mu$A/cm$^2$; SD 24). To conserve tissues, we tested each gallbladder preparation from PAR knockout mice with peptide agonists followed by proteases at 10 min intervals without intervening washes.

Gallbladders from PAR\textsubscript{1}\textsuperscript{-/-} mice were completely non-responsive to both TFLLRN-NH\textsubscript{2} and thrombin, whereas the $I_{sc}$ responses to SLIGRL-NH\textsubscript{2} and trypsin were maintained (Fig. 6). Similarly, gallbladders from PAR\textsubscript{2}\textsuperscript{-/-} mice were non-responsive to SLIGRL-NH\textsubscript{2}, but retained a small response to trypsin, probably due to trypsin activation of PAR\textsubscript{1} (7; 45; 49), and responded normally to TFLLRN-NH\textsubscript{2} and thrombin (Fig. 7). Thus, thrombin and trypsin stimulate $I_{sc}$ by activating PAR\textsubscript{1} and PAR\textsubscript{2}, respectively.

Ion substitution reduces electrogenic responses to activation of PAR\textsubscript{1} and PAR\textsubscript{2}. To determine the ionic basis for PAR-induced stimulation of $I_{sc}$, we substituted HCO$_3^\text{-}$ or Cl$^-$ ions.

Removal of HCO$_3^\text{-}$ ions from either serosal or luminal buffers decreased the response to thrombin by 96% and 83%, respectively (serosal removal: $I_{sc} = 2.0\mu$A/cm$^2$ (SD 3), n=3, P=0.02; luminal removal: $I_{sc} = 8.7\mu$A/cm$^2$; SD 5.7; n=3; P=0.04) (Fig. 8A). Similarly, removal of HCO$_3^\text{-}$ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH\textsubscript{2} by 89% and 69%, respectively (serosal removal: $I_{sc} = 7.3\mu$A/cm$^2$ (SD 5.7), n=3, P=0.009; luminal
removal: $I_{sc} = 22 \mu A/cm^2$ (SD 10), n=3, P=0.03). Therefore, activation of PAR$_1$ caused electrogentic conductance that depends on the presence of HCO$_3^-$ in both luminal and serosal buffers.

Removal of HCO$_3^-$ from the serosal buffer decreased the response to trypsin by 83% ($I_{sc} = 10.3 \mu A/cm^2$, SD 8.3, n=3, P=0.006) (Fig. 9B). However, removal of HCO$_3^-$ from the luminal buffer did not affect the trypsin-induced $I_{sc}$ response ($I_{sc} = 52\mu A/cm^2$, SD 22), n=3, P=0.63). Similarly, removal of HCO$_3^-$ from the serosal buffer decreased responses to SLIGRL-NH$_2$ by 79% (8.5 $\mu A/cm^2$ (SD 5.4), n=3, P=0.02), but removal of HCO$_3^-$ from the luminal buffer had no effect (48 $\mu A/cm^2$ (SD 33.5), n=5, P=0.84) (Fig. 9B). Thus, PAR$_2$ activation induces primarily HCO$_3^-$ conduction (secretion) in a serosal to luminal direction, and PAR$_2$-induced anion secretion does not require luminal HCO$_3^-$ ions.

To examine a possible Cl$^-$ dependence of either PAR$_1$- or PAR$_2$-activated $I_{sc}$ responses, Cl$^-$ ions were removed from serosal and luminal buffers. Removal of Cl$^-$ ions from both serosal and luminal compartments caused a 96% decrease in TFLLRN-NH$_2$-stimulated $I_{sc}$ ($I_{sc}=4.1\mu A/cm^2$ (SD 2.2), n=4, P=0.002). Similarly, removal of Cl$^-$ ions from both compartments caused a 97% decrease in SLIGRL-NH$_2$-stimulated gallbladders ($I_{sc}=1.1 \mu A/cm^2$ (SD 1.2), n=4, P=0.007). Current recordings were consistently unstable when we removed Cl$^-$ ions from either
side alone. Nevertheless, our results indicate that activation of both PAR\textsubscript{1} and PAR\textsubscript{2} results in an electrogenic response that is dependent on Cl\textsuperscript{-} conduction.

**PAR\textsubscript{1} and PAR\textsubscript{2} signaling to GBEC in culture.** In the intact gallbladder, our results show that proteases and APs regulate anion secretion. These effects may be mediated by PARs that are expressed by epithelial cells, which prominently express PAR\textsubscript{1} and PAR\textsubscript{2} (Fig. 2). To directly determine if proteases can signal to epithelial cells by cleaving PARs, we isolated epithelial cells from the mouse gallbladder. Cultured GBEC were prominently stained with an antibody to cytokeratin 19, confirming their epithelial nature (Fig. 9). We investigated whether isolated GBEC expressed functional PAR\textsubscript{2} by measuring [Ca\textsuperscript{2+}] mobilization. [Ca\textsuperscript{2+}] was measured in individual cells using Fura2/AM. PAR\textsubscript{1}AP (100 \mu M) and PAR\textsubscript{2}AP (100 \mu M) stimulated increases in [Ca\textsuperscript{2+}] (Fig. 10). (PAR\textsubscript{1}AP was \sim 10 fold less potent than PAR\textsubscript{2}AP (EC\textsubscript{50} \sim 10 nM), and there was no Ca\textsuperscript{2+} response to the corresponding reverse peptide sequences (data not shown). These results confirm functional expression of PAR\textsubscript{1} and PAR\textsubscript{2} on isolated epithelial cells.

**Mechanism of PAR\textsubscript{1}- and PAR\textsubscript{2}-stimulated $I_{\text{sc}}$.** Previous studies have shown that the CFTR is a dominant pathway for anion secretion in the gallbladder (13; 34). Therefore, we used CFTR inh\textsubscript{172}, a potent and selective inhibitor of the CFTR (31), to test the hypothesis that CFTR is the anion channel triggered by PARs. As a first step, we verified the inhibitory effect of the CFTR inh\textsubscript{172} on forskolin-stimulated tissue. Forskolin (10 \mu M) caused a rapid and sustained ($\sim$ 10 min)}
increase in $I_{sc}$ from baseline (Fig. 11A). Application of CFTR inh172 (5 µM) to the luminal buffer two minutes after forskolin stimulation caused an immediate reduction in $I_{sc}$ by 85% (Fig. 11B).

Similarly, PAR$_1$ activation by TFLLRN-NH$_2$ induced a normal magnitude increase in $I_{sc}$, and application of CFTR inh172 two minutes after stimulation caused an immediate and pronounced decrease in $I_{sc}$ by 75% (Fig. 11C). A similar reduction in current by CFTR inh172 was observed following PAR$_1$ activation by thrombin (not shown). In contrast, application of CFTR inh172 two minutes after stimulation of PAR$_2$ by SLIGRL-NH$_2$ or trypsin had no effect on $I_{sc}$ (data not shown). These findings strongly suggest that PAR$_1$ stimulates anion secretion through the apically located CFTR channel in mouse gallbladder, whereas PAR$_2$ does not. These findings are further supported by studies using NPPB, a nonspecific Cl$^-$ channel blocker (that may inhibit the CFTR anion channel) (21). Application of NPPB (100 µM) resulted in a transient increase in $I_{sc}$ that quickly returned to baseline. NPPB completely inhibited PAR$_1$ activation by PAR$_1$AP. However, incubation with NPPB caused a decrease in the $I_{sc}$ response to PAR$_2$AP by 39% compared to control ($I_{sc}$=28.2 µA/cm$^2$ SD 7.7, p=0.07, n=5) (Fig 12).

In lung and other gastrointestinal tissues, prostaglandins play an important role in PAR-mediated regulation of electrolyte function. PAR$_2$ activation causes arachidonic acid release and prostaglandin formation, which are required for PAR$_2$-stimulated Isc (27; 40; 44). Therefore, we
examined the effects of indomethacin, a non-selective cyclooxygenase (COX) inhibitor on \( I_{sc} \).

Indomethacin (10 \( \mu \)M) caused a slight increase in \( I_{sc} \) baseline (~5 \( \mu A/cm^2 \)) but completely inhibited the \( I_{sc} \) response to thrombin and TFLLRN-NH$_2$ (Fig. 13A). In contrast, stimulation of PAR$_2$ with trypsin or SLIGRL-NH$_2$ in the presence of indomethacin resulted in \( I_{sc} \) responses that were normal in magnitude (Fig. 13A). Ketorolac (10 \( \mu \)M), a relatively selective COX-1 inhibitor, caused a decrease in PAR$_1$ activation by 15% (n=4) without affecting the PAR$_2$ response (data not shown). Meloxicam (1 \( \mu \)M), which has a 300-fold selectivity for COX-2 vs. COX-1 (23), caused complete inhibition of PAR$_1$ activation, also without affecting PAR$_2$ activity (Fig. 13B).

Thus, PAR$_1$ stimulation in gallbladder tissue is dependent on eicosanoid formation and COX-2 activation, whereas of PAR$_2$ is not.

A luminal Ca$^{2+}$-mediated anion conductance that is independent of CFTR has been described.(2; 14; 35) Therefore, we used DIDS, which blocks Ca$^{2+}$-activated chloride channels, to examine the possibility that a Ca$^{2+}$-mediated conductance accounted for the effects of PAR agonists. Pre-incubation with DIDS (500 \( \mu \)M) added to the luminal buffer or added two minutes after PAR stimulation had no effect on normal \( I_{sc} \) responses to TFLLRN-NH$_2$ or SLIGRL-NH$_2$ (data not shown). Thus, PAR-stimulated anion conduction does not involve Ca$^{2+}$-activated Cl$^-$ channels.
Pretreatment of gallbladders with bumetanide (100 μM) or furosemide (1 mM), inhibitors of the Na⁺-K⁺-2Cl⁻ cotransporter system, did not affect the Isc responses to PAR₁ or PAR₂ peptide agonists (data not shown), suggesting that the Na⁺-K⁺-2Cl⁻ transporter is not required. Thus, the strong inhibitory effect of Cl⁻ ion removal may be related to cell shrinkage or disruption or an unidentified ion transport process.

Amiloride (10 μM), an inhibitor of the epithelial sodium channel, had no effect on the Isc baseline. However, PAR₁ and PAR₂ Isc responses were consistently increased in magnitude in the presence of amiloride. Serosal activation of PAR₁ by TFLLRN-NH₂ caused an increase in the Isc response that was 1.6 times greater than normal in the presence of amiloride (113 μA/cm² (SD 26.8), P=0.1, n=5). Similarly, PAR₂ AP generated Isc responses that were 2.2 times greater than normal in the presence of amiloride (121 μA/cm² (SD 15.7), P = 0.0001, n=5. This apparent increase in PAR-stimulated anion secretion could be explained by the presence of an apical sodium channel that is weakly sensitive (does not affect baseline Isc) to amiloride that blocks depolarizing conductance, thereby causing a hyperpolarization of the cell, which could increase the driving force for apical anion secretion.

Because PAR₁ and PAR₂ have been shown to mediate ion secretion through neurogenic pathways (9; 20), we also evaluated electrogenic current responses in the presence of tetrodotoxin (neuronal conduction blocker). Pre-treatment with tetrodotoxin (0.1 μM) had no
effect on baseline $I_{sc}$, nor did it alter $I_{sc}$ responses to PAR$_1$ or PAR$_2$ activation, thereby excluding a neurogenic mechanism (data not shown).

**DISCUSSION**

In this study, we used a combination of molecular, immunohistochemical and functional approaches to detect PARs in the gallbladder and to examine their role in regulating electrolyte secretion. Our results show that PAR$_1$ and PAR$_2$ are expressed in the normal mouse gallbladder, where they are prominently localized to epithelial cells. Application of proteases and selective agonists of PAR$_1$ and PAR$_2$ to the serosal surface of the gallbladder stimulates $I_{sc}$. Observations in desensitized tissues and from knockout mice show that thrombin stimulates $I_{sc}$ by activating PAR$_1$, and that trypsin stimulates $I_{sc}$ by activating PAR$_2$. PAR$_1$-stimulated $I_{sc}$ depends on the presence of HCO$_3^-$ ions in the luminal and serosal buffers, whereas PAR$_2$-stimulated $I_{sc}$ depends on the presence of HCO$_3^-$ ions only in the serosal buffer. Both responses also require Cl$^-$ ions. The effects of PAR$_1$ agonists depend on the CFTR channel, generation of eicosanoids, and COX-2 activation, and may thus be indirect. PAR$_2$ agonists do not require prostaglandin generation and may be direct. These newly characterized mechanisms of protease-regulated electrogenic secretion in the gallbladder may contribute to altered functions of the inflamed gallbladder, when PAR-activating proteases are generated and released.
PAR expression and function in the gallbladder. Evidence for the presence of PAR₁ and PAR₂ in gallbladder tissue is limited to observations that agonists affect motility by a prostanoid-dependent mechanism in the guinea-pig (43). We confirmed the expression of PAR₁ and PAR₂ in the mouse gallbladder by several approaches. First, we amplified PAR₁ and PAR₂ mRNA from the mouse gallbladder using RT-PCR and purified and sequenced the products to confirm their identity. Second, we localized immunoreactive PAR₁ and PAR₂ in the mouse gallbladder using immunohistochemistry, and found prominent expression of both receptors in epithelial cells. Third, we obtained functional evidence for the expression of PAR₁ and PAR₂ in the epithelium since selective peptides and PAR-activating proteases stimulated anion secretion. Fourth, to directly determine if agonists can signal to epithelial cells by activating PARs, we isolated and cultured GBEC and measured increases in [Ca²⁺]. In support of our findings, PAR₁ and PAR₂ are prominently expressed by other epithelial cells in the digestive tract, including the small intestine, colon and pancreatic duct, where activation regulates prostaglandin generation, transepithelial ion transport and paracellular permeability (12; 17; 25; 27; 36; 44).

PAR₁ and PAR₂ regulation of Iₛₑ in the gallbladder. Because the primary function of the gallbladder is to actively modify bile volume and composition, we examined the role of PARs in regulating epithelial fluid and electrolyte absorption and secretion in this tissue. The results of our study suggest that serosal application of thrombin, trypsin, PAR₁ and PAR₂ selective peptide
agonists (TFLLRN-NH₂ and SLIGRL-NH₂, respectively) stimulate anion secretion, probably by activating receptors on the basolateral surface of epithelial cells. When applied to the apical surface, proteases induced a small increase in I_sc, whereas APs had no effect. One explanation for this lack response to luminal application of APs may be due to the presence of membrane bound (non-specific) proteases that may degrade and inactivate these peptides. Others have reported similar findings, showing that PAR peptide agonists mainly stimulate transepithelial ion transport when applied to the basolateral surface (3; 17; 28; 33; 36; 40). We observed that challenge of tissues with selective agonists of PAR₁ and PAR₂ desensitized the I_sc responses to thrombin and trypsin, respectively, suggesting that thrombin and trypsin stimulate I_sc by activating PAR₁ and PAR₂. As expected, proteases produced stronger desensitized responses to second challenges than did the corresponding selective peptide agonists. This more complete desensitization of PAR₁ and PAR₂ by proteases is probably due to the permanent physical changes in the receptors that occur with proteolysis, making them unresponsive to an additional stimulus(5). Moreover, the ability of proteases to activate multiple receptors may explain why desensitization in I_sc was not observed when proteases were applied after APs. To confirm this suggestion, we studied gallbladders from PAR₁- and PAR₂-deficient mice. Our results show that tissues from PAR₁⁻/⁻ mice responded normally to PAR₂ agonists, and vice versa.
Mechanisms of PAR\textsubscript{1} and PAR\textsubscript{2}-mediated electrogenic secretion. We investigated the mechanism by which PAR\textsubscript{1} and PAR\textsubscript{2} activation affects transepithelial secretion by ion substitution, and by using agonists and antagonists of various secretory mechanisms. Our results show that activation of PARs stimulates anion secretion based on the direction of the current generated and the insensitivity to amiloride. Both bicarbonate and chloride are the transported ions, since removal of these anions prevented PAR-stimulated $I_{sc}$.

Chloride ions may also be transported across the apical membrane of gallbladder epithelial cells. Previous studies in murine gallbladder have reported successful unilateral Cl\textsuperscript{−} substitution (from either luminal or serosal surfaces alone) (13; 41). We were unable to reproduce these conditions because of unstable current recordings. However, when Cl\textsuperscript{−} was removed from both gallbladder surfaces, PAR\textsubscript{1}- and PAR\textsubscript{2}-stimulated $I_{sc}$ was almost abolished. A likely explanation for this requirement of Cl\textsuperscript{−} ions for PAR-stimulated $I_{sc}$ is the presence of a HCO\textsubscript{3}\textsuperscript{−}/Cl\textsuperscript{−} exchanger on the apical membrane, where luminal Cl\textsuperscript{−} ions would be necessary for HCO\textsubscript{3}\textsuperscript{−} secretion across the apical membrane.

The CFTR Cl\textsuperscript{−} channel is required for cAMP-dependent secretion of HCO\textsubscript{3}\textsuperscript{−} ions in the gallbladder since tissues from CFTR\textsuperscript{−/−} mice show no $I_{sc}$ response to forskolin (13; 34). We found that the CFTR antagonism by CFTR\textsuperscript{inh172} strongly inhibited PAR\textsubscript{1}-stimulated $I_{sc}$, but not PAR\textsubscript{2}, indicating that this response requires CFTR. Moreover, Cl\textsuperscript{−} channel Inhibitor studies using NPPB
completely inhibited PAR_1 induced anion secretion, and decreased PAR_2 AP activity by 39%,
supporting the concept that NPPB blocks the CFTR Cl^- channel (21).

PAR_1-and PAR_2-stimulated current was prevented by removal of HCO_3^- ions from the
serosal buffer. When HCO_3^- was removed from the luminal buffer, normal magnitude I_{sc}
responses were observed with PAR_2 activation, whereas PAR_1-stimulated I_{sc} was strongly
inhibited. These results suggest that PAR_1 and PAR_2 agonists stimulate secretion of HCO_3^- ions
in the mouse gallbladder. In support of HCO_3^- being the major transported anion, previous
studies on murine gallbladder epithelia showed that electrogenic HCO_3^- rather than Cl^- secretion
occurs in a serosal to luminal direction by a forskolin-stimulated cAMP pathway (18; 34).
However, several of our findings suggest that PAR_1 and PAR_2 stimulate HCO_3^- ion secretion by
distinct mechanisms. First, the responses to PAR_1 agonists required luminal HCO_3^- whereas the
response to PAR_2 agonists did not. Second, indomethacin and meloxicam completely inhibited
the I_{sc} response to PAR_1 agonists, but did not affect the I_{sc} response to PAR_2 agonists. Thus,
whereas PAR_2 agonists probably stimulate ion secretion directly in the mouse gallbladder,
agonists of PAR_1 may induce the generation of prostaglandins by epithelial or perhaps other cell
types in the gallbladder, and indirectly stimulate ion secretion. Third, PAR_1-stimulated anion
secretion in mouse gallbladder is mediated by CFTR, but PAR_2 is not. In contrast to our results,
others have shown that PAR_2-mediated ion transport requires prostaglandin synthesis in
intestinal and lung epithelia (27; 33; 40; 44), suggesting a variable role of PARs in electrolyte transport in different tissues and species. Moreover, as previously proposed for airway epithelia (40), anion secretion depends on prostaglandin release and CFTR activation. Our results are in agreement with this model except that in mouse gallbladder tissue, the transported anion is primarily HCO₃⁻, and the activated receptor is PAR₁, and not PAR₂.

**Potential physiological and pathophysiological roles of PARs in the gallbladder.**

Proteases that can activate PARs in the gallbladder may arise from normal gastrointestinal secretions (trypsins), or from the circulation (e.g., coagulation factors) and inflammatory cells (e.g., mast cell proteases) during inflammation. Under normal circumstances, pancreatic proteases may also be present in the gallbladder since during the interdigestive period, the sphincter at the duodenum is closed, thereby shunting bile flow into the relaxed gallbladder. These proteases might participate in the physiological regulation of anion secretion through PAR₁ or PAR₂, thereby influencing bile output and composition. Indeed, PAR₂ agonists stimulate fluid and electrolyte secretion in small intestine and colon, which may be protective by diluting and removing luminal toxins (17; 27).

PARs-stimulated secretion in the gallbladder may also be protective during cholecystitis, since the gallbladder mucosa, like other gastrointestinal tissues, is injured by direct exposure to conjugated and unconjugated bile salts. In disease, gallstones obstruct at the sphincter of Oddi
and may divert pancreatic proteases into the biliary tree, thereby modulating inflammation. High trypsin concentrations in gallbladder bile have been documented in patients with common bile duct stones (1; 46; 47). During inflammation there are elevated levels of proteases from inflammatory cells (mast cells, tryptase, neutrophil-derived proteases), the epithelium (trypsins) and the circulation (coagulation factors). Certainly, our results support the concept that interstitially derived proteases are the likely mechanism involved in regulating anion secretion in the gallbladder since activation of PARs by agonists applied to the luminal surface was limited. Further investigations are necessary to define the role of PARs in the gallbladder in health and disease states.
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44. **Vergnolle N, Macnaughton WK, Al Ani B, Saifeddine M, Wallace JL and Hollenberg MD.** Proteinase-activated receptor 2 (PAR2)-activating peptides: identification of a


**FIGURE LEGENDS**

**Figure 1. Detection of PAR₁ and PAR₂ in whole gallbladder by RT-PCR.** (A) A 305 bp product corresponding to PAR₁ and a 330 bp product corresponding to PAR₂ were detected. (B) The 371 bp product corresponding to PAR₄ was detected in mouse myocardium, but not found in gallbladder tissue. The lower second band from myocardium corresponded to the sequence of Mus musculus MYST histone acetyltransferase monocytic leukemia 4. There were no products when RT was omitted.
Figure 2. Localization of PAR₁ and PAR₂ in gallbladder by immunohistochemistry. (A) Immunoreactive PAR₁ was detected in epithelial cells (small arrow) as well as the serosa and muscular layer (large arrow). (B) Immunoreactive PAR₂ was detected in the epithelium (small arrow) and muscle layer (large arrow). (C, D) Preabsorption with the receptor fragments used for immunization abolished the signals. Scale bar = 32 μm.

Figure 3. Effects of proteases (A) and peptide agonists of PAR₁ (B) on Iₛₑ in gallbladder. Gallbladders were exposed to agonists on the serosal or luminal surfaces at the indicated concentrations (arrows) and the Iₛₑ (µA/cm²) was measured. When applied to the serosal surface, thrombin and TFLLRN-NH₂ stimulated Iₛₑ (upper panels), but boiled thrombin and NRLLFT-NH₂ had no effect (middle panels). When applied to the luminal surface, thrombin but not TFLLRN-NH₂ stimulated Iₛₑ (lower panels). Traces are from single mice and are representative of experiments on >3 mice.

Figure 4. Effects of proteases (A) and peptide agonists of PAR₂ (B) on Iₛₑ in gallbladder. Gallbladders were exposed to agonists on the serosal or luminal surfaces at the indicated concentrations (arrows) and the Iₛₑ (µA/cm²) was measured. When applied to the serosal surface, trypsin and SLIGRL-NH₂ stimulated Iₛₑ (upper panels), but boiled trypsin and LRGILS-NH₂ had no effect (middle panels). When applied to the luminal surface, trypsin but not SLIGRL-NH₂ stimulated Iₛₑ (lower panels). Traces are from single mice and are representative of experiments on >3 mice.

Figure 5. Desensitization of Iₛₑ in gallbladder in response to repeated application of agonists to PAR₁ (A) and PAR₂ (B). Gallbladders were exposed sequentially to agonists at the indicated concentrations (arrows) without an intervening wash, and transepithelial current was measured. (A) Thrombin strongly desensitized responses to TFLLRN-NH₂ (upper panel) and abolished responses to a second challenge with thrombin (A, second panel). When TFLLRN-
NH₂, was used as the first agonist, the response to a second equimolar challenge with TFLLRN-NH₂ was desensitized by 53% relative to the initial Iₛₛ response. TFLLRN-NH₂ also desensitized tissues to a second challenge with thrombin by 71% (A, lower panels). **(B)** Trypsin abolished responses to SLIGRL-NH₂ (upper panel) and desensitized responses to a second challenge with trypsin (B, second panel). SLIGRL-NH₂ also desensitized responses to a second challenge with SLIGRL-NH₂, but did not desensitize the Iₛₛ response to a second challenge with trypsin (B, lower panels). Traces are from single animals and are representative of experiments on >3 mice.

**Figure 6. The effects of PAR agonists on Iₛₛ in gallbladder from PAR₁⁻/⁻ mice.** PAR₁ AP, TFLLRN-NH₂, and thrombin had no effect on Iₛₛ, but SLIGRL-NH₂ and trypsin activation did. Forskolin was used to confirm tissue viability. Traces are from single mice and are representative of experiments on 3 mice.

**Figure 7. The effects of PAR agonists on Iₛₛ in gallbladder from PAR₂⁻/⁻ mice.** PAR₂ AP, SLIGRL-NH₂, had no effect on Iₛₛ, but TFLLRN-NH₂ and thrombin activation did. A small trypsin response is seen, most likely due to trypsin’s ability to activate PAR₁. Forskolin was used to confirm tissue viability. The trace is from a single animal and is representative of experiments of 3 mice.

**Figure 8. Effects of HCO₃⁻ removal from serosal or luminal compartments.** PAR₁-induced secretion was inhibited by removal of both serosal or luminal HCO₃ (A). PAR₂-induced secretion was inhibited by removal of serosal but not luminal HCO₃ (B). * P<0.05 vs control.
Figure 9. (A) Phase contrast image of isolated and cultured gallbladder epithelial cells. (B) Fluorescent microscopy image—arrow points to epithelial cell stained with an antibody to cytokeratin 19. Scale bar = 50 µM (A), 20 µM (B)

Figure 10. Effects of PAR APs on \([\text{Ca}^{2+}]_i\) in cultured GBECs. The left panels show the time course of results from individual epithelial cells. The right panel shows the pseudocolor images of the same cells as those in the left panel. * Indicates the time at which these images were taken (40 and 25 s).

Figure 11. CFTR inhibition. Normal \(I_{sc}\) response to forskolin (A). Luminal CFTRinh-172 inhibition of \(I_{sc}\) two minutes after stimulation by forskolin (B) and PAR\(_1\) agonist (C). Traces are from single mice and are representative of experiments on 3 mice.

Figure 12. Effects of NPPB on \(I_{sc}\) and activation of PARs. Application of NPPB caused a transient increase in \(I_{sc}\) and blocked responses the PAR\(_1\) agonist (TFLLRN-NH\(_2\)). The \(I_{sc}\) response to PAR\(_2\) AP (SLIGRL-NH\(_2\)) was reduced by 39% compared to control. The trace is from a single animal and is representative of experiments on 5 mice.

Figure 13. Effects of COX inhibitors on \(I_{sc}\) and activation of PARs. (A) Indomethacin treatment did not affect responses to the PAR\(_2\) agonists trypsin and SLIGRL-NH\(_2\), but abolished \(I_{sc}\) responses to the PAR\(_1\) agonists TFLLRN-NH\(_2\) and thrombin. (B) Meloxicam caused complete inhibition of PAR\(_1\) activation, also without affecting PAR\(_2\) activity. The trace is from a single animal and is representative of experiments on 6 mice.
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Figure 4. Effects of proteases (A) and peptide agonists of PAR2 (B) on Isc in gallbladder. Gallbladders were exposed to agonists on the serosal or luminal surfaces at the indicated concentrations (arrows) and the Isc (μA/cm²) was measured. When applied to the serosal surface, trypsin and SLIGRL-NH₂ stimulated Isc (upper panels), but boiled trypsin and LRGILS-NH₂ had no effect (middle panels). When applied to the luminal surface, trypsin but not SLIGRL-NH₂ stimulated Isc (lower panels). Traces are from single mice and are representative of experiments on >3 mice.
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Figure 7. The effects of PAR agonists on Isc in gallbladder from PAR2-/- mice. PAR2 AP, SLIGRL-NH2, had no effect on Isc, but TFLLRN-NH2 and thrombin activation did. A small trypsin response is seen, most likely due to trypsin's ability to activate PAR1. Forskolin was used to confirm tissue viability. The trace is from a single animal and is representative of experiments of 3 mice.
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