Agonists of protease-activated receptors 1 and 2 stimulate electrolyte secretion from mouse gallbladder

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Kirkland JG, Cottrell GS, Bunnett NW, Corvera CU. Agonists of protease-activated receptors 1 and 2 stimulate electrolyte secretion from mouse gallbladder. Am J Physiol Gastrointest Liver Physiol 293: G335–G346, 2007. First published April 12, 2007; doi:10.1152/ajpgi.00425.2006.—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 PAR1 and PAR2 to the epithelium, muscle, and serosa of mouse gallbladder. mRNA transcripts corresponding to PAR1 and PAR2, but not PAR4, were detected by RT-PCR and sequencing. Addition of thrombin and a PAR1-selective activating peptide (TFLLRN-NH2) to the serosal surface of mouse gallbladder mounted in an Ussing chamber stimulated an increase in short-circuit current in wild-type but not PAR1 knockout mice. Similarly, serosally applied trypsin and PAR2 activating peptide (SLIGRL-NH2) increased short-circuit current in wild-type but not PAR2 knockout mice. Proteases and activating peptides strongly inhibited electrogenic responses to subsequent stimulation with the same agonist, indicating homologous desensitization. Removal of HCO3− ions from the serosal buffer reduced responses to thrombin and trypsin by >80%. Agonists of PAR1 and PAR2 increase intracellular Ca2+ concentration in isolated and cultured gallbladder epithelial cells. The COX-2 inhibitor meloxicam and an inhibitor of CFTR prevented the stimulatory effect of PAR1 but not PAR2. Thus PAR1 and PAR2 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.

bicarbonate transport; epithelial transport

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, 16). Kallikreins are able to activate PAR1, PAR2, and PAR4 (38). Proteases cleave within the extracellular NH2-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 PAR1 stimulate Cl− secretion in the intestinal epithelium (8), and agonists of PAR2 also regulate the epithelial cells of the digestive tract to stimulate generation of prostaglandins in the intestine (27), promote secretion of Cl− 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 (GBEC), where they control ion transport. Our aims were to 1) examine the expression and localization of PARs in the mouse gallbladder by 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, 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). PAR agonists (forward, 5'-TGCTGTATCCTTTGGTGCTG-3'; reverse, 5'-TGGGAGGTATCACCCT-3') were chosen to amplify a 330-bp fragment. Primers to mouse PAR 4 (GGAAGGCTGAC-3') and mouse PAR2 (SLIGRL-NH2) and their respective reverse sequences (NRLIFS-NH2, NRLFTT-NH2, and LRGILS-NH2), which were used as inactive controls, were from Genemed Synthesis (South San Francisco, CA). TFLLRN-NH2 is highly selective for PAR1, whereas SFLRN-NH2 is not (22). 2-Thiocyano-thiazolidine 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). The GenBank accession number for PAR1 is NM_010169 and for PAR2 is NM_007974.

Mice. Male CD-1 mice (4–6 mo, 35–45 g) were from Charles River (Willington, MA) 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 were given water ad libitum. Mice were killed by 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 by using Trizol (Invitrogen, Carlsbad, CA). Genomic DNA was eliminated by using DNase I (Ambion, Austin, TX). RNA (1.0 μg) was reverse transcribed by using the AMV-RT system (Promega, Madison, WI), and cDNA was amplified with Taq polymerase (Invitrogen). Primers to mouse PAR1 (forward, 5'-CTATGAGCGCCAGCAATC-3'; reverse, 5'-ACGTCCAAGGAAGGCTGAC-3') were chosen to amplify a 305-bp fragment. Primers to mouse PAR2 (forward, 5'-TGGGAGGTATCACCCT-TCTG-3'; reverse, 5'-CCAGGGTTCGCTGATGACTC-3') were chosen to amplify a 330-bp fragment. Primers to mouse PAR4 (forward, 5'-TGCTGTATCCTTTGGTGCTG-3'; reverse, 5'-CATGGCAGAATGGTGTTAGT-3') were chosen to amplify an expected product size of 373 bp. The PCR conditions were denaturation for 10 min at 94°C, 35 cycles of 94°C for 30 s, 55°C for 30 s, 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 h at 4°C. Gallbladders were embedded in optimum cutting temperature compound, and frozen sections (8 μm) were prepared. Tissue sections were rehydrated in PBS (pH 7.4) for 15 min and were incubated in blocking buffer by using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). 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 diamobenzene peroxide substrate wash solution (Vector Laboratories) were used for detection according to the manufacturer’s instructions. For controls, PAR1 and PAR2 antibodies were preabsorbed with the receptor fragment used for immunization (10 μM, 72 h, 4°C).

Isolation and culture of GBEC. GBEC were isolated by using a previously described procedure (29). Briefly, gallbladders (minimum of 5) were dissected free of connective tissue and placed in ice-cold culture medium. They were opened, rinsed with cold PBS, and placed in prewarmed (37°C) trypsin + EDTA, PBS for 45 min. The released GBEC were collected, filtered, and washed with MEM +10% fetal calf serum (EMEM). Cells were centrifuged, resuspended in insulin Transferin-Selenium-Supplemented EMEM, and plated onto 15-mm, circular, collagen-coated glass coverslips for measurement of intracellular Ca2+ mobilization ([Ca2+]i) and for cytokeratin staining (Santa Cruz Biotechnology).

Measurement of [Ca2+]i. GBEC were isolated and cultured for 7–14 days. GBEC were incubated in PBS containing 0.1% BSA, 7 μM 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 Fluor ×40 objective (numerical aperture 1.3). Agonists were added directly to the chamber from concentrated stock solutions. Images were obtained by using an integrated charge-coupled device 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 [Ca2+]i, was calculated. Fresh GBEC were studied with each agonist concentration, and a minimum of 15 GBEC were analyzed from 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 slides (surface area 0.031 cm2) and were transferred to Ussing-style chambers for continuous measurement of short-circuit current (Isc) by using a VCC MC6 clamp system (EasyMount diffusion chambers; Physiological Instruments, San Diego, CA). Electrode agar bridges were made with 3–4% agarose in 3 M KCl saturated with Ag and AgCl2. The Isc was determined by clamping trans epithelial voltage to 0 mV by using the voltage-clamp mode of the amplifier. An increase in Isc may be due to either electrogenic cation absorption, anion secretion, or some combination of both. Tissues were bathed in KBS containing (in mM) 120 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.8 K2HPO4, 1.2 MgCl2, 1.2 CaCl2, and 5 glucose at 37°C, pH 7.3, and were bubbled with 95% O2-5% CO2. Data were acquired by using the Biopac data-acquisition system (Biopac, Santa Barbara, CA). 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 nM; CaCl2, 300 μM; forskolin, 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 Isc 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. HCO3−-free solutions were made by substituting NaHCO3 and KH2PO4 with sucrose and adding 10 mM HEPES buffer. Cl−-free solutions were made by substituting NaCl with Na-glucocate.
Statistical analysis. Data are presented as means (SD). Tissues from at least three mice were used in all experiments. Comparisons between a single control and treatment mean were made by using the unpaired, two-tailed Student’s 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) whole gallbladder tissue by RT-PCR. Products of anticipated active PAR1 was localized to GBEC from the mucosa (Fig. 2A) (42). The purified PCR products were sequenced to confirm their identity. The sequenced PCR product from the gallbladder, but it was detected in mouse myocardium used as a positive control (Fig. 1A) (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 GBEC from the mucosa (Fig. 2A). 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). Staining by both antibodies was abolished by preabsorption with the receptor fragments used for immunization, confirming specificity (Fig. 2, C and D). 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 \( I_{sc} \). To determine the role of PAR1 and PAR2 in regulating electrolyte secretion and to confirm functional expression of PARs, we measured changes in \( I_{sc} \) to graded concentrations of agonists in whole gallbladder tissues. The baseline \( I_{sc} \) was 17.4 \( \mu A/cm^2 \) (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 \( I_{sc} \) of 52.2 \( \mu A/cm^2 \) (SD 30.6) over baseline \((n = 8)\) that returned to prestimulated levels by 5 min (Fig. 3A, top). Luminal (apical) treatment with thrombin stimulated smaller increases in \( I_{sc} \) [35.4 \( \mu A/cm^2 \) (SD 23.8); \( n = 4 \)] that were slow in onset and sustained (Fig. 3A, bottom). Heat-inactivated thrombin had no effect (Fig. 3A, middle). The peptide agonist corresponding to the tethered ligand of human PAR1, SFLLRN-NH2 (100 \( \mu M \)) also increased \( I_{sc} \) by 54.0 \( \mu A/cm^2 \) (SD 48.2) \((n = 12)\), but only when applied to the serosal surface (not shown). Because SFLLRN-NH2 also activates PAR2 (4), we assayed the PAR2-selective analog TFLLRN-NH2. Serosal stimulation with TFLLRN-NH2 (100 \( \mu M \)) caused a rapid increase in \( I_{sc} \) of 71.2 \( \mu A/cm^2 \) (SD 31.1) \((n = 7)\) (Fig. 3B, top) with a similar efficacy but reduced potency to thrombin \((EC_{50} \sim 50 \mu M; \) Fig. 5A). However, TFLLRN-NH2 had no effect when applied to the luminal surface (Fig. 3B, bottom).

The corresponding reverse sequences of the tethered ligand peptides RNLLFT-NH2 (Fig. 3B, middle) 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 $I_{sc}$ when added to either serosal or luminal surfaces at the indicated concentrations (arrows), and the $I_{sc}$ (μA/cm²) was measured. When applied to the serosal surface, thrombin (A, top) and TFLLRN-NH₂ (B, top) stimulated $I_{sc}$, but boiled thrombin (A, middle) and NRLLFT-NH₂ (B, middle) had no effect. When applied to the luminal surface, thrombin (A, bottom) but not TFLLRN-NH₂ (B, bottom) stimulated $I_{sc}$. Traces are from single mice and are representative of experiments on >3 mice.

Accordingly, PAR agonists were added only to the serosal surface in subsequent experiments. The $I_{sc}$ 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, top). 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 $I_{sc}$ response. TFLLRN-NH₂ also desensitized tissues to a second challenge with thrombin by 71% (SD 22) ($n = 5$; Fig. 5A, bottom). 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, top). SLIGRL-NH₂ desensitized tissues to a second challenge with SLIGRL-NH₂ by 55% (SD 16). However, SLIGRL-NH₂ did not desensitize the I_{sc} response to a second challenge with trypsin (Fig. 5B, bottom), 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 PAR 2 in gallbladder tissue.

Thrombin and trypsin stimulate I_{sc} by activating PAR1 and PAR2, respectively. Receptor desensitization by selective agonists suggested that proteases affect electrolyte secretion by activating PAR1 and PAR2. However, because proteases can have many actions, we studied their effects on I_{sc} in PAR-deficient mice to confirm the mechanisms of protease-stimulated electrolyte secretion. Because both PAR1 and PAR2 knockout mice were bred to a C57Bl/6 background, we first evaluated the wild-type mice for functional expression of PAR1 and PAR2 as a necessary control. In wild-type C57Bl/6 mice, PAR1 and PAR2 agonists stimulated normal I_{sc} responses [PAR1 AP, TFLLRN-NH₂, 51.0 μA/cm² (SD 38.0); PAR2 AP, SLIGRL-NH₂, 51.0 μA/cm² (SD 24.0)]. 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 PAR1-deficient mice were completely nonresponsive to both TFLLRN-NH₂ and thrombin, whereas the I_{sc} responses to SLIGRL-NH₂ and trypsin were maintained (Fig. 6). Similarly, gallbladders from PAR2-deficient mice were nonresponsive to SLIGRL-NH₂ but retained a small response to trypsin, probably due to trypsin activation of PAR1 (7, 45, 49), and responded normally to TFLLRN-NH₂ and thrombin (Fig. 7). Thus thrombin and trypsin stimulate I_{sc} by activating PAR1 and PAR2, respectively.

Fig. 4. Effects of proteases (A) and peptide agonists of PAR2 (B) on I_{sc} in gallbladder. Gallbladders were exposed to agonists on the serosal or luminal surfaces at the indicated concentrations (arrows), and the I_{sc} (μA/cm²) was measured. When applied to the serosal surface, trypsin (A, top) and SLIGRL-NH₂ (B, top) stimulated I_{sc}, but boiled trypsin (A, middle) and LRGILS-NH₂ (B, middle) had no effect. When applied to the luminal surface, trypsin (A, bottom) but not SLIGRL-NH₂ (B, bottom) stimulated I_{sc}. Traces are from single mice and are representative of experiments on >3 mice.
Ion substitution reduces electrogenic responses to activation of PAR1 and PAR2. To determine the ionic basis for PAR-induced stimulation of Iₑ, we substituted HCO₃⁻/H₁₁₀₀₂ or Cl⁻/H₁₁₀₀₂ ions. Removal of HCO₃⁻/H₁₁₀₀₂ ions from either serosal or luminal buffers decreased the response to thrombin by 96% and 83%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A]. Similarly, removal of HCO₃⁻ ions from either the serosal or luminal KBS reduced the electrogenic current to TFLLRN-NH₂ by 89% and 69%, respectively [serosal removal: Iₑ = 2.0 μA/cm² (SD 3.0), n = 3, P = 0.02; luminal removal: Iₑ = 8.7 μA/cm² (SD 5.7), n = 3, P = 0.04; Fig. 8A].
Mechanism of PAR1- and PAR2-stimulated $I_{sc}$. Previous studies have shown that the CFTR is a dominant pathway for anion secretion in the gallbladder (13, 34). Therefore, we used CFTRinh-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 CFTRinh-172 on forskolin-stimulated tissue. Forskolin (10 μM) caused a rapid and sustained (>10 min) increase in $I_{sc}$ from baseline (Fig. 11A). Application of CFTRinh-172 (5 μM) to the luminal buffer 2 min after forskolin stimulation caused an immediate reduction in $I_{sc}$ by 85% (Fig. 11B). Similarly, PAR1 activation by TFFLRN-NH$_2$ induced a normal-magnitude increase in $I_{sc}$, and application of CFTRinh-172 2 min after stimulation caused an immediate and pronounced decrease in $I_{sc}$ by 75% (Fig. 11C). A similar reduction in current by CFTRinh-172 was observed following PAR1 activation by thrombin (not shown). In contrast, application of

![Diagram](image_url)

Fig. 7. Effects of PAR agonists on $I_{sc}$ in gallbladder from PAR2 knockout mice. PAR2 AP TFFLRN-NH$_2$ had no effect on $I_{sc}$, but TFFLRN-NH$_2$ 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. Trace is from a single animal and is representative of experiments on 3 mice.

![Diagram](image_url)

Fig. 8. Effects of HCO$_3^-$ removal from serosal or luminal compartments. PAR$_1$-induced secretion was inhibited by removal of both serosal or luminal HCO$_3^-$ (A). PAR$_2$-induced secretion was inhibited by removal of serosal but not luminal HCO$_3^-$ (B). *$P < 0.05$ vs. control.
CFTRinh-172 2 min after stimulation of PAR2 by SLIGRL-NH2 or trypsin had no effect on $I_{sc}$ (data not shown). These findings strongly suggest that PAR1 stimulates anion secretion through the apically located CFTR channel in mouse gallbladder, whereas PAR2 does not. These findings are further supported by studies using NPPB, a nonspecific Cl⁻/H⁺-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 PAR1 activation by PAR1 AP. However, incubation with NPPB caused a decrease in the $I_{sc}$ response to PAR2 AP by 39% compared with control [$I_{sc} = 28.2 \mu A/cm^2$ (SD 7.7), $n = 5$, $P = 0.07$; Fig. 12].

In lung and other gastrointestinal tissues, prostaglandins play an important role in PAR-mediated regulation of electrolyte function. PAR2 activation causes arachidonic acid release and prostaglandin formation, which are required for PAR2-stimulated $I_{sc}$ (27, 40, 44). Therefore, we examined the effects of indomethacin, a nonselective COX inhibitor, on $I_{sc}$. Indomethacin (10 μM) caused a slight increase in $I_{sc}$ baseline (~5 μA/cm²) but completely inhibited the $I_{sc}$ response to thrombin and TFLLRN-NH₂ (Fig. 13A). In contrast, stimulation of PAR2 with trypsin or SLIGRL-NH₂ in the presence of indomethacin resulted in $I_{sc}$ responses that were normal in magnitude (Fig. 13A). Ketorolac (10 μM), a relatively selective COX-1 inhibitor, caused a decrease in PAR1 activation by 15% ($n = 4$) without affecting the PAR2 response (data not shown). Meloxicam (1 μM), which has a 300-fold selectivity for COX-2 vs. COX-1 (23), caused complete inhibition of PAR1 activation, also without affecting PAR2 activity (Fig. 13B). Thus PAR1 stimulation in gallbladder tissue is dependent on eicosanoid formation and COX-2 activation, whereas stimulation of PAR2 is not.

A luminal Ca²⁺-mediated anion conductance that is independent of CFTR has been described (2, 14, 35). Therefore, we used DIDS, which blocks Ca²⁺-activated Cl⁻ channels, to examine the possibility that a Ca²⁺-mediated conductance accounted for the effects of PAR agonists. Preincubation with DIDS (500 μM) added to the luminal buffer or added 2 min after PAR stimulation had no effect on normal $I_{sc}$ responses to TFLLRN-NH₂ or SLIGRL-NH₂ (data not shown). Thus PAR-stimulated anion conduction does not involve Ca²⁺-activated Cl⁻ channels.

Pretreatment of gallbladders with bumetanide (100 μM) or furosemide (1 mM), inhibitors of the Na⁺-K⁺-2Cl⁻ cotrans-

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Fig. 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 bars: 50 μM (A), 20 μM (B).

Fig. 10. Effects of PAR APs on intracellular Ca²⁺ concentration in cultured gallbladder epithelial cells. Left: time course of results from individual epithelial cells. Right: pseudocolor images of the same cells. * Indicates the time at which these images were taken (40 and 25 s).

Fig. 11. CFTR inhibition. Normal $I_{sc}$ response to forskolin (A), luminal CFTR inhibitor (CFTRinh-172) inhibition of $I_{sc}$ 2 min after stimulation by forskolin (B), and response to PAR1 agonist (C) are shown. Traces are from single mice and are representative of experiments on 3 mice.
porter system, did not affect the $I_{sc}$ 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 to an unidentified ion-transport process.

Amiloride (10 μM), an inhibitor of the epithelial Na$^{+}$ channel, had no effect on the $I_{sc}$ baseline. However, PAR1 and PAR2 $I_{sc}$ responses were consistently increased in magnitude in the presence of amiloride. Serosal activation of PAR1 by TFLLRN-NH$_2$ caused an increase in the $I_{sc}$ response that was 1.6 times greater than normal in the presence of amiloride [113.0 μA/cm$^2$ (SD 26.8), $n$ = 5, $P$ = 0.011]. Similarly, PAR2 AP generated $I_{sc}$ responses that were 2.2 times greater than normal in the presence of amiloride [121.0 μA/cm$^2$ (SD 15.7), $n$ = 5, $P$ = 0.0001]. This apparent increase in PAR-stimulated anion secretion could be explained by the presence of an apical Na$^{+}$ channel that is weakly sensitive (does not affect baseline $I_{sc}$) 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 electrogeneic current responses in the presence of tetrodotoxin (neuronal conduction blocker). Pretreatment with tetrodotoxin (0.1 μM) had no effect on baseline $I_{sc}$, nor did it alter $I_{sc}$ responses to PAR1 or PAR2 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 PAR1 and PAR2 are expressed in the normal mouse gallbladder, where they are prominently localized to GBEC. Application of proteases and selective agonists of PAR1 and PAR2 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 PAR1 and that trypsin stimulates $I_{sc}$ by activating PAR2. PAR1-stimulated $I_{sc}$ depends on the presence of HCO$_3^-$ ions in the luminal and serosal buffers, whereas PAR2-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 PAR1 agonists depend on the CFTR channel, generation of eicosanoids, and COX-2 activation and may thus be indirect. PAR2 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 by using RT-PCR and purified and sequenced the products to confirm their identity. Second, we localized immunoreactive PAR1 and PAR2 in the mouse gallbladder by using immunohistochemistry and found prominent expression of both receptors in GBEC. Third, we obtained functional evidence for the expression of PAR1 and PAR2 in the epithelium, because selective peptides and PAR-activating proteases stimulated anion secretion. Fourth, to directly determine whether agonists can signal to GBEC by activating PARs, we isolated and cultured GBEC and measured increases in $[Ca^{2+}]_i$. In support of our findings, PAR1 and PAR2 are prominently expressed by other epithelial

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**Fig. 12.** Effects of 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) on $I_{sc}$ and activation of PARs. Application of NPPB caused a transient increase in $I_{sc}$ and blocked responses the PAR agonist (TFLLRN-NH$_2$). The $I_{sc}$ response to PAR2 AP (SLIGRL-NH$_2$) was reduced by 39% compared with control. Trace is from a single animal and is representative of experiments on 5 mice.

**Fig. 13.** Effects of COX inhibitors on $I_{sc}$ and activation of PARs. A: indomethacin treatment did not affect responses to the PAR1 agonists trypsin and SLIGRL-NH$_2$ but abolished $I_{sc}$ responses to the PAR2 agonist TFLLRN-NH$_2$ and thrombin. B: meloxicam caused complete inhibition of PAR1 activation, also without affecting PAR2 activity. Trace is from a single animal and is representative of experiments on 6 mice.
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 $I_{sc}$ 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, and PAR1- and PAR2-selective peptide agonists (TFLLRN-NH$_2$ and SLIGRL-NH$_2$, respectively) stimulate anion secretion, probably by activating receptors on the basolateral surface of GBEC. When applied to the apical surface, proteases induced a small increase in $I_{sc}$, whereas APs had no effect. One explanation for this lack of response to luminal application of APs may be due to the presence of membrane-bound (nonspecific) 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 $I_{sc}$ response 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 knockout mice responded normally to PAR2 agonists and vice versa.

**Mechanisms of PAR1- and PAR2-mediated electrogenic secretion.** We investigated the mechanism by which PAR1 and PAR2 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 according to the direction of the current generated and the insensitivity to amiloride. Both HCO$_3^-$ and Cl$^-$ are the transported ions, because removal of these ions prevented PAR-stimulated $I_{sc}$.

Cl$^-$ ions may also be transported across the apical membrane of GBEC. Previous studies in murine gallbladder have reported successful unilateral Cl$^-$ 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$^-$ was removed from both gallbladder surfaces, PAR1- and PAR2-stimulated $I_{sc}$ was almost abolished. A likely explanation for this requirement of Cl$^-$ ions for PAR-stimulated $I_{sc}$ is the presence of a HCO$_3^-$/Cl$^-$ exchanger on the apical membrane, where luminal Cl$^-$ ions would be necessary for HCO$_3^-$ secretion across the apical membrane.

The CFTR Cl$^-$ channel is required for cAMP-dependent secretion of HCO$_3^-$ ions in the gallbladder, because tissues from CFTR$^{-/-}$ mice show no $I_{sc}$ response to forskolin (13, 34). We found that the CFTR antagonism by CFTRinh-172 strongly inhibited PAR1-stimulated $I_{sc}$ but not PAR2, indicating that this response requires CFTR. Moreover, Cl$^-$-channel inhibitor studies using NPPB completely inhibited PAR1-induced anion secretion and decreased PAR2 AP activity by 39%, supporting the concept that NPPB blocks the CFTR Cl$^-$ channel (21). PAR1- and PAR2-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 PAR2 activation, whereas PAR1-stimulated $I_{sc}$ was strongly inhibited. These results suggest that PAR1 and PAR2 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 PAR1 and PAR2 stimulate HCO$_3^-$ ion secretion by distinct mechanisms. First, the responses to PAR1 agonists required luminal HCO$_3^-$, whereas the response to PAR2 agonists did not. Second, indomethacin and meloxicam completely inhibited the $I_{sc}$ response to PAR1 agonists but did not affect the $I_{sc}$ response to PAR2 agonists. Thus, whereas PAR2 agonists probably stimulate ion secretion directly in the mouse gallbladder, agonists of PAR1 may induce the generation of prostaglandins by epithelial or perhaps other cell types in the gallbladder and thereby indirectly stimulate ion secretion. Third, PAR1-stimulated anion secretion in mouse gallbladder is mediated by CFTR, but PAR2 is not. In contrast to our results, others (27, 33, 40, 44) have shown that PAR2-mediated ion transport requires prostaglandin synthesis in intestinal and lung epithelia, 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 HCO$_3^-$ and the activated receptor is PAR1, 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, because 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).

PAR-stimulated secretion in the gallbladder may also be protective during cholecystitis, because 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 (trypsin), 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, because 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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