Extracellular UTP stimulates electrogenic bicarbonate secretion across CFTR knockout gallbladder epithelium

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Clarke, Lane L., Matthew C. Harline, Lara R. Gawenis, Nancy M. Walker, John T. Turner, and Gary A. Weisman. Extracellular UTP stimulates electrogenic bicarbonate secretion across CFTR knockout gallbladder epithelium. Am J Physiol Gastrointest Liver Physiol 279: G132–G138, 2000.—The loss of cystic fibrosis transmembrane conductance regulator (CFTR)-mediated transepithelial HCO₃⁻ secretion contributes to the pathogenesis of pancreatic and biliary disease in cystic fibrosis (CF) patients. Recent studies have investigated P2Y₂ nucleotide receptor agonists, e.g., UTP, as a means to bypass the CFTR defect by stimulating Ca²⁺-activated Cl⁻ secretion. However, the value of this treatment in facilitating transepithelial HCO₃⁻ secretion is unknown. Gallbladder mucosae from CFTR knockout mice were used to isolate the Ca²⁺-dependent anion secretion in these tissues, and, recently, it was shown (24) that the CFTR primarily mediates electrogenic HCO₃⁻ secretion across the murine gallbladder. Therefore, to isolate the effect of UTP on Ca²⁺-mediated HCO₃⁻ secretion and to simulate the CF condition, studies using the pH stat techn-


**MATERIAL AND METHODS**

**Animals.** Weanling mice (2–4 mo of age) born to animals heterozygous for the disrupted murine homologue of the *cftr* gene (B6.129-Cftrtm1.UNC, C57BL/6J-Cftrtm1.UNC) were used. The genotype of each littermate was determined using a PCR technique employing primers specific for murine *cftr* and the neomycin resistance/*cftr* junction, as previously described (7). CFTR(−/−) and CFTR(+/+) mice were homozygous for the disrupted *cftr* gene and the wild-type *cftr* gene, respectively. The mice were fed standard laboratory mouse chow and water ad libitum until the evening before an experiment and then only drinking water was provided. The drinking water for all mice contained an osmotic laxative (polyethylene glycol) to prevent intestinal impaction in the CFTR(−/−) mice (7). The University of Missouri-Columbia Institutional Animal Care and Use Committee approved all experiments involving the animals.

**In vitro bioelectric and pH stat measurements.** Mice were killed on the day of the experiment by brief exposure to an atmosphere of 100% CO₂ to induce basal narcosis, followed by a surgically induced bilateral pneumothorax. The gallbladder and a portion of surrounding liver were excised en masse via an abdominal incision and immediately placed in ice-cold, oxygenated Ringer solution (containing 1 mM indomethacin to prevent prostanoid generation). Under a dissecting microscope, the gallbladder was dissected free of hepatic tissue, opened longitudinally, and placed mucosal-side up on the stage of a dissecting microscope, the gallbladder was dissected free of hepatic tissue, opened longitudinally, and placed mucosal-side up on the stage of a dissecting microscope. The serosal bath was mounted horizontally in a modified Ussing chamber (0.126 or 0.238 cm² exposed surface area), and Parafilm “O” rings were used to minimize edge damage where the gallbladder was secured between chamber halves.

The bioelectric and pH stat studies were performed as recently described (9). The gallbladder preparations were bathed on the luminal surface with an unbuffered Ringer (NaCl) solution that was gassed with 100% O₂ and contained (in mM) 143.8 NaCl, 5.2 KCl, 1.2 CaCl₂, and 1.2 MgCl₂. The serosal surface was bathed with a standard Krebs-Ringer-bicarbonate (KRB) solution gassed with 95% O₂-5% CO₂ and containing (in mM) 115 NaCl, 2.4 K₂HPO₄, 0.4 KH₂PO₄, 25 NaHCO₃, 1.2 CaCl₂, 1.2 MgCl₂, and 10 glucose (pH 7.4). In some experiments, Cl⁻ was replaced with an equimolar concentration of gluconate⁻ (3 mM CaSO₄ was added to overcome Ca²⁺ chelation). The solutions were circulated throughout the experiment by gas lift and warmed to 37°C by water-jacketed reservoirs. Before each experimental protocol, gallbladders were equilibrated for 20 min under short-circuit conditions with TTX (0.1 μM) in the serosal bath to minimize variation due to intrinsic neural tone.

Transepithelial short-circuit current (I\textsubscript{s-c}, in μeq · cm⁻² · h⁻¹) was measured using an automatic voltage clamp (VCC-600, Physiologic Instruments, San Diego, CA) and calomel electrodes that were connected to the chambers by 4% agar-3 M KCl bridges, as previously described (9). I\textsubscript{s-c} and automatic fluid resistance compensation current were applied through Ag-AgCl electrodes connected to the chamber baths via 4% agar-NaCl bridges. Every 5 min during an experiment, a 5-mV pulse was passed across the gallbladder tissue to determine the total tissue conductance (G\textsubscript{t}, mS/cm² tissue surface area) by measuring the magnitude of the resulting current deflections and applying Ohm’s law. The serosal bath served as ground in all experiments.

The serosal-to-mucosal flux of HCO₃⁻ (J\textsubscript{s-m}, in μeq · cm⁻² · h⁻¹) was measured by pH stat titration of the luminal bath (4 ml) to pH 7.4 using 5 mM HCl delivered by either a computer-aided titrimeter (Fisher, model 455/465) or manual addition of titrant. The volume of added acid was used to calculate the HCO₃⁻ (base) flux, taking into account the time period and the surface area of the tissue. Typically, I\textsubscript{s-c} stabilized within 30 min after the tissue was mounted, and the luminal solution was replaced to refresh transepithelial ion gradients and remove secreted mucus. A 30-min basal flux period was initiated, and then UTP (100 μM) was added to the luminal bath. I\textsubscript{s-c} assumed a plateau phase after 5 min, and a second 30-min flux period was initiated.

**Statistics.** Student’s paired or unpaired t-tests were used for statistical comparisons. P ≤ 0.05 was considered statistically significant. Unless otherwise indicated, data are presented as means ± SE.

**MATERIALS.** Immediately before each experiment, a stock solution of 10 mM UTP (Boehringer-Mannheim, Indianapolis, IN) was made in NaCl and titrated to pH 7.4. Indomethacin (Sigma Chemical, St. Louis, MO) was dissolved in DMSO at a stock concentration of 0.01 M. DISD (Aldrich Chemical, Milwaukee, WI) was dissolved in NaCl solution at a stock concentration of 0.03 M. TTX (Sigma Chemical) was dissolved in 0.2% acetic acid at a stock concentration of 0.0001 M. All other reagents were obtained from either Sigma Chemical, Aldrich Chemical, or Fisher Scientific (Springfield, NJ).

**RESULTS**

**Ussing chamber studies.** Previous studies (10) of murine gallbladder epithelium established the presence of a luminal membrane P₂Y₁ receptor that is stimulated by UTP and results in Ca²⁺-activated, transepithelial anion secretion. In normal murine gallbladder, CFTR is a basally active anion conductance that has been shown to mediate both electrogenic Cl⁻ and HCO₃⁻ secretion (24). Because the presence of CFTR complicates the analysis of Ca²⁺-activated anion currents, studies evaluating the ionic basis of the UTP-stimulated I\textsubscript{s-c} were performed on freshly excised gallbladder epithelium from CFTR knockout mice. As shown in Fig. 1, cAMP-dependent stimulation of CFTR(+/+) gallbladders by a forskolin treatment induced a large I\textsubscript{s-c} response, whereas treatment of the CFTR(−/−) gallbladders was essentially without effect. In contrast, UTP treatment of the luminal membrane stimulated a nearly equivalent I\textsubscript{s-c} response in both CFTR(+/+) and CFTR(−/−) gallbladders. Previous Ussing chamber studies of murine gallbladder have established that 100 μM UTP induces a near-maximal I\textsubscript{s-c} response when added to the luminal bath but is relatively ineffective when added to the serosal bath (10).

An I\textsubscript{s-c} recording trace of the UTP response in a CFTR(−/−) gallbladder epithelium with NaCl in the luminal bath and KRB in the serosal bath is shown in Fig. 2A. Typical of Ca²⁺-activated transepithelial anion secretion, the I\textsubscript{s-c} after UTP treatment immediately increased to a maximum value and then decreased within minutes to a stable plateau where it remained elevated over baseline. The plateau I\textsubscript{s-c} in the UTP-treated CFTR(−/−) gallbladder typically was elevated for over 30 min. Subsequent treatment of the gallbladder...
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Fig. 1. Comparison of the maximal change in short-circuit current (I_{sc}) after treatment with either 10 μM forskolin (Forsk) or 100 μM UTP in gallbladder epithelium from cystic fibrosis transmembrane conductance regulator (CFTR(+/+)) and CFTR(−/−) mice (n = 5 each). ΔI_{sc} has been normalized to 1 cm² of mucosal surface area. *Significantly different from CFTR(+/+).

In contrast to the Na⁺-K⁺-2Cl⁻ cotransporter, did not affect the I_{sc} suggesting that the plateau I_{sc} was not due to Cl⁻ secretion. To evaluate the Cl⁻ dependence of the UTP-induced I_{sc} response, CFTR(−/−) gallbladders were bathed in identical solutions except that Cl⁻ was replaced with gluconate⁻. As shown in Fig. 2B, the time course of the immediate I_{sc} response to UTP was reduced in the Cl⁻-free Ringer solution, whereas the plateau I_{sc} phase was not affected. These findings are consistent with the hypothesis that the plateau phase of the UTP-induced I_{sc} response in CFTR(−/−) gallbladder epithelium represents electrogenic HCO₃⁻ secretion. To evaluate the HCO₃⁻ dependence of the sustained I_{sc} response, CFTR(−/−) gallbladders were bathed in HCO₃⁻-free Ringer solution (TES buffered, gassed with 100% O₂) and treated with 100 μM methazolamide (luminal + serosal) to inhibit endogenous HCO₃⁻ production (Fig. 2C). This condition markedly attenuated the initial I_{sc} response and abolished the I_{sc} plateau after UTP treatment. Next, we asked whether the plateau I_{sc} after UTP could be inhibited at the luminal membrane with DIDS (300 μM), a blocker of the Ca²⁺-activated anion conductance (4, 8). As shown in Fig. 2D, DIDS treatment completely abolished the plateau I_{sc} response after UTP addition. DIDS at 100 μM concentration also completely inhibited the plateau phase of the UTP-induced I_{sc} response (data not shown). As a control, 300 μM DIDS was added to the serosal bath after UTP and did not decrease but, unexpectedly, caused an increase in the I_{sc} (+2.3 and +1.9 μA, n = 2).

pH stat studies. The preceding findings were consistent with the hypothesis that the plateau phase of the I_{sc} response after UTP treatment represents electrogenic HCO₃⁻ secretion via a Ca²⁺-activated anion conductance in murine gallbladder. Therefore, the I_{sc} and J_{sc} were simultaneously measured using the pH stat technique (both parameters are expressed in μeq · cm⁻² · h⁻¹ for comparison). The flux studies performed on intact CFTR(−/−) gallbladders consisted of two periods, a 30-min basal flux period followed by addition of luminal UTP and a 30-min treatment flux period (i.e., during the plateau phase of the I_{sc} response to luminal UTP). As shown in Fig. 3, the baseline J_{sc} across CFTR(−/−) gallbladder was 4.31 ± 0.64 μeq · cm⁻² · h⁻¹ and the I_{sc} was 2.04 ± 0.28 μeq · cm⁻² · h⁻¹. During the plateau phase beginning 5 min after luminal UTP addition, the J_{sc} increased by 0.84 ± 0.21 μeq · cm⁻² · h⁻¹ which was nearly equivalent to the stable increase in I_{sc} of 0.79 ± 0.32 μeq · cm⁻² · h⁻¹. Luminal UTP addition did not alter the G, between the two flux periods, indicating that changes in the conductance of the paracellular pathway did not contribute to the increased rate of HCO₃⁻ secretion (basal Gₛ = 44.4 ± 5.3; UTP Gₛ = 47.0 ± 6.1 mS/cm²). To evaluate whether direct manipulation of Ca²⁺ mobilization would generate a response similar to that of UTP, additional experiments were performed using the Ca²⁺ ionophore ionomycin (1 μM) added to the luminal bath of CFTR(−/−) gallbladders. Ionomycin treatment initially caused a rapid J_{sc} and I_{sc} response similar to UTP, but the plateau phase only lasted ~20 min. However, both J_{sc} and I_{sc} were significantly increased during the 20-min period after ionomycin treatment (ΔJ_{sc} = 1.1 ± 0.3 μeq · cm⁻² · h⁻¹, P < 0.05; ΔI_{sc} after ionomycin = 1.7 ± 0.6 μeq · cm⁻² · h⁻¹, P < 0.05; ΔI_{sc} 20 min after ionomycin = 0.4 ± 0.2 μeq · cm⁻² · h⁻¹, not significant; n = 4).

Previous studies have provided evidence that cAMP-dependent stimulation of normal murine gallbladder induces electrogenic HCO₃⁻ secretion via a CFTR-dependent process (24). For purposes of comparison with the UTP response, we measured the effect of cAMP-dependent stimulation on J_{sc} and I_{sc} in intact CFTR(+/+). UTP (10 μM) treatment to increase intracellular cAMP, the ΔJ_{sc} and ΔI_{sc} were 1.76 ± 0.42 and 1.98 ± 0.47 μeq · cm⁻² · h⁻¹, respectively, over basal values (basal J_{sc} and I_{sc} were 5.18 ± 0.66 and 1.41 ± 0.27 μeq · cm⁻² · h⁻¹, respectively; n = 6). Thus the plateau phase of the response to UTP treatment in a CFTR(−/−) gallbladder yields a ΔJ_{sc} that is 48% and a ΔI_{sc} that is 40% of the responses to forskolin treatment in CFTR(+/+). The nearly equivalent increases in ΔJ_{sc} and ΔI_{sc} after UTP treatment suggest a process of electrogenic HCO₃⁻ secretion mediated by a Ca²⁺-activated HCO₃⁻ conductance. However, it is also possible that UTP stimulates a Ca²⁺-activated Cl⁻ conductance that increases the activity of a luminal membrane Cl⁻/HCO₃⁻ antiporter system (28). To investigate this possibility, CFTR(−/−) gallbladders were bathed with a Cl⁻-free solution in the luminal bath to inhibit activity of luminal membrane Cl⁻/HCO₃⁻ exchange. As shown in Fig. 4, substitution of luminal Cl⁻ with gluconate⁻ greatly...
reduced the basal $J_{\text{s-m}}$, indicating that Cl⁻/HCO₃⁻ exchange contributes significantly to basal HCO₃⁻ secretion. The basal $I_{\text{sc}}$ in the absence of luminal Cl⁻ was reversed in polarity, largely as a result of uncompensated junction potentials. Nevertheless, UTP treatment in the absence of luminal Cl⁻ stimulated increases in $J_{\text{s-m}}$ and $I_{\text{sc}}$ that were similar to the UTP responses in luminal NaCl solution ($\Delta J_{\text{s-m}} = 1.13 \pm 0.23 \mu\text{eq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$; $\Delta I_{\text{sc}} = 1.34 \pm 0.35 \mu\text{eq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$). UTP treatment under these conditions had no effect on $G_t$ (basal $G_t = 19.8 \pm 3.1 \text{mS/cm}^2$; UTP $G_t = 18.7 \pm 2.7 \text{mS/cm}^2$).

We observed that treatment with luminal DIDS abolishes the plateau phase of the $I_{\text{sc}}$ response (Fig. 2), consistent with inhibition of the Ca²⁺-activated anion conductance (4). Therefore, we asked whether luminal DIDS treatment would inhibit UTP-dependent stimulation of both $J_{\text{s-m}}$ and $I_{\text{sc}}$ during the plateau phase of the response. As shown in Fig. 5, luminal treatment with DIDS (300 µM) during the plateau phase of the

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Fig. 2. Recordings showing the effect of different conditions on $I_{\text{sc}}$ in UTP-stimulated (100 µM) gallbladder epithelium from CFTR(−/−) mice. A: $I_{\text{sc}}$ response in gallbladder epithelium bathed with NaCl Ringer solution on the luminal surface and Krebs-Ringer-bicarbonate (KRB) solution on the serosal surface. Bumetanide (Bumet; 100 µM) was added to the serosal bath (arrow). Basal $I_{\text{sc}} = 7.7 \mu\text{A}$ (mean = $6.4 \pm 1.7 \mu\text{A}$ or $1.67 \pm 0.44 \mu\text{eq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$, $n = 3$). B: $I_{\text{sc}}$ response in gallbladder epithelium bathed bilaterally with Cl⁻/HCO₃⁻ exchange. The basal $I_{\text{sc}}$ in the absence of luminal Cl⁻ was reversed in polarity, largely as a result of uncompensated junction potentials. Nevertheless, UTP treatment in the absence of luminal Cl⁻ stimulated increases in $J_{\text{s-m}}$ and $I_{\text{sc}}$ that were similar to the UTP responses in luminal NaCl solution ($\Delta J_{\text{s-m}} = 1.13 \pm 0.23 \mu\text{eq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$; $\Delta I_{\text{sc}} = 1.34 \pm 0.35 \mu\text{eq} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$). UTP treatment under these conditions had no effect on $G_t$ (basal $G_t = 19.8 \pm 3.1 \text{mS/cm}^2$; UTP $G_t = 18.7 \pm 2.7 \text{mS/cm}^2$).

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UTP response eliminated both the increases in $J_{s\rightarrow m}$ and $I_{sc}$ in the CFTR(−/−) gallbladder. The $G_t$ was not different between the basal and UTP treatment periods under these conditions (basal $G_t = 30.1 \pm 5.7$ mS/cm², UTP $G_t = 29.0 \pm 3.4$ mS/cm²). The ability of luminal DIDS to block both the $I_{sc}$ and $J_{s\rightarrow m}$ is consistent with the conclusion that $Ca^{2+}_{l}$-activated anion conductance was responsible for the Cai mobilization, and $I_{sc}$ (10). Characterization studies showed that the peak $I_{sc}$ response to UTP is independent of luminal Na⁺, DIDS sensitive, reduced by removal of either Cl⁻ or HCO₃⁻ from the medium, and almost completely abolished by removal of both anions. With the use of CFTR knockout mice in the present study, $I_{sc}$ measurements resulting from activation of the $Ca^{2+}_{l}$-mediated anion conductance were uncomplicated by CFTR activity, a major conductive pathway for cAMP-mediated electrogenic anion secretion in the murine gallbladder (24, 29). In support of using the CF murine gallbladder for isolation of the $Ca^{2+}_{l}$-activated anion channel conductance, an epithelial $Ca^{2+}_{l}$-activated Cl⁻ channel (mCLCA) has recently been cloned and was shown to be expressed at high levels in murine gallbladder (15, 17). Whether the mCLCA channel is solely responsible for the $Ca^{2+}_{l}$-activated anion channel activity demonstrated in earlier studies (26) of biliary epithelium has yet to be determined.

Two models have been proposed for electrogenic HCO₃ secretion across epithelial tissues. In one model, an inward current is generated by HCO₃⁻ that permeates anion channels in the luminal membrane (1, 12). In a second model, the inward current is generated by Cl⁻ permeation of luminal membrane anion channels. Cl⁻ secretion in the latter model facilitates HCO₃ secretion by “recycling” Cl⁻ entering via a luminal membrane Cl⁻/HCO₃ exchanger (28). Several lines of evidence in the present study indicate that the plateau phase of the UTP-induced $I_{sc}$ response represents a HCO₃ secretory current carried by the $Ca^{2+}_{l}$-activated anion conductance (model 1). First, the plateau $I_{sc}$ response to UTP was not inhibited by bumetanide or complete Cl⁻ substitution but required HCO₃⁻ in the bathing medium. Second, the rates of UTP-induced $J_{s\rightarrow m}$ and $I_{sc}$ were similar in magnitude both under control conditions and during inhibition of Cl⁻/HCO₃ exchange.
exchange by luminal Cl− removal. Third, the UTP-stimulated increase in \( J_{sc} \) and \( I_{sc} \) during the plateau phase of the response could be completely inhibited by micromolar DIDS, a known blocker of the Ca\(^{2+}\)-activated anion conductance (4). Whether the recently cloned mCLCA channel mediates this HCO\(_3\) conductance is not yet known. To the best of our knowledge, neither the mCLCA channel nor the Ca\(^{2+}\)-activated anion channels described in biliary epithelial cells (15, 26) have been evaluated for HCO\(_3\) permeation.

Luminal Cl− removal also revealed a significant amount of Cl−/HCO\(_3\) exchange in the basal state of the CF murine gallbladder [for comparison, electroneutral HCO\(_3\) secretion in the mucous duodenum is less than one-third of this rate (9)]. If the residual \( J_{sc} \) in the absence of luminal Cl− is taken as a measure of net paracellular flow of HCO\(_3\) (−1 μeq · cm\(^{-2} \) · h\(^{-1} \)), then the basal \( J_{sc} \) of the murine gallbladder would be ~3 and 4 μeq · cm\(^{-2} \) · h\(^{-1} \) in CF and wild-type mice, respectively. These values compare reasonably well with the basal \( J_{sc} \) measured across the gallbladder of rabbit and guinea pig (~3 and ~2 μeq · cm\(^{-2} \) · h\(^{-1} \), respectively) (30, 35). Note, however, that the direction of net HCO\(_3\) movement (absorptive vs. secretory) across murine gallbladder in the basal state could not be ascertained because mucosal-to-serosal flux of HCO\(_3\) was not measured. Nonetheless, Cl−/HCO\(_3\) exchange apparently dominates the basal secretory flux of HCO\(_3\) in the mouse gallbladder as it does in rabbit and guinea pig (30, 35). Although further studies will be necessary to determine the molecular identity of luminal Cl−/HCO\(_3\) exchange, it was apparent from the studies shown in Fig. 5 that electroneutral HCO\(_3\) secretion was not highly sensitive to luminal DIDS (~12% decrease, not significant). This observation suggests that weakly DIDS-sensitive exchangers, such as the downregulated in adenoma protein (murine homologue) and the anion exchanger AE2 (2, 27), are candidate proteins for the luminal Cl−/HCO\(_3\) exchange process in murine gallbladder.

In summary, it was shown that P2Y\(_{2}\)-receptor activation of a Ca\(^{2+}\)-mediated anion conductance results in electroneutral HCO\(_3\) secretion across CF murine gallbladder epithelium. This finding suggests that nucleotide receptor therapy may restore in CF patients the loss of regulated HCO\(_3\) secretion that is necessary for transgluminal pH modulation in certain organs. In comparison to CFTR-mediated HCO\(_3\) secretion in murine gallbladder, ~50% of this activity can be induced by UTP treatment. However, the duration of the response is relatively short lived (30–60 min). Efforts to prolong activation of this pathway, perhaps by preventing P2Y\(_{2}\)-receptor desensitization (10), may be beneficial. As previously proposed for murine airway epithelia (8), the dominance of the Ca\(^{2+}\)-activated anion conductance in other murine epithelial tissues may be responsible for the relative lack of pancreatic and biliary disease in CF mouse models. Thus increasing the activity and expression of components of the Ca\(^{2+}\)-activated anion conductance pathway in human epithelia is a potential treatment for cystic fibrosis.

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