MEMBRANE TRANSPORT STRUCTURE FUNCTION AND BIOGENESIS:

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Ca^{2+} Activates Cystic Fibrosis Transmembrane Conductance Regulator- and Cl^--dependent HCO_3^- Transport in Pancreatic Duct Cells*

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Pancreatic duct cells secrete bicarbonate-rich fluids, which are important for maintaining the patency of pancreatic ductal trees as well as intestinal digestive function. The bulk of bicarbonate secretion in the luminal membrane of duct cells is mediated by a Cl^--dependent mechanism (Cl^-/HCO_3^- exchange), and we previously reported that the mechanism is CFTR-dependent and cAMP-activated (Lee, M. G., Choi, J. Y., Luo, X., Strickland, E., Thomas, P. J., and Muallem, S. (1999) *J. Biol. Chem.* 274, 14670–14677). In the present study, we provide comprehensive evidence that calcium signaling also activates the same CFTR- and Cl^--dependent HCO_3^- transport. ATP and trypsin evoked intracellular calcium signaling in pancreatic duct-derived cells through the activation of purinergic and protease-activated receptors, respectively. Cl^-/HCO_3^- exchange activity was measured by recording pH_i in response to $[Cl^-]_o$ changes of the perfusate. In perfusate containing high concentrations of K^+, which blocks Cl^- movement through electrogenic or K^+-coupled pathways, ATP and trypsin highly stimulated luminal Cl^-/HCO_3^- exchange activity in CAPAN-1 cells expressing wild-type CFTR, but not in CFPAC-1 cells that have defective (ΔF508) CFTR. Notably, adenoviral transfection of wild-type CFTR in CFPAC-1 cells completely restored the stimulatory effect of ATP on luminal Cl^-/HCO_3^- exchange. In addition, the chelation of intracellular calcium by 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*′-tetraacetic acid (BAPTA) treatment abolished the effect of calcium agonists on luminal Cl^-/HCO_3^- exchange. These results provide a molecular basis for calcium-induced bicarbonate secretion in pancreatic duct cells and highlight the importance of CFTR in epithelial bicarbonate secretion induced by various stimuli.

It is well established that pancreatic HCO_3^- secretion performs a critical role in digestion. HCO_3^- protects duodenal epithelia by neutralizing gastric acid and maintains an optimum intraluminal pH for digestive enzymes (1). Accumulating evidence suggests that HCO_3^--rich pancreatic fluid secretion is also important for maintaining the patency of intrapancreatic ductal trees (2–4). Transepithelial HCO_3^- transport is the principal driving force for fluid secretion by pancreatic duct cells, and reduced HCO_3^- concentrations result in acidification of the luminal environment. The rheologic properties of mucus are critically affected by mucin concentrations and the pH of solvent. In general, mucin precipitation and viscosity are progressively increased as the pH and the volume of secreted fluid decrease (5, 6). Indeed, the most prominent changes observed in the pancreatic juice of obstructive ductal diseases, such as cystic fibrosis (CF)[1] or chronic pancreatitis, are reductions in secreted volume and HCO_3^- concentration (2, 7).

Although there is some disagreement on the precise mechanism of ductal HCO_3^- secretion, it is widely accepted that the bulk of HCO_3^- secretion is mediated by Cl^-/HCO_3^- exchange on the luminal membrane (8). Previously, we showed that cystic fibrosis transmembrane conductance regulator (CFTR) activates Cl^-/HCO_3^- exchange activity in heterologous expression systems and in the luminal membrane of pancreatic duct (9, 10). Notably, the CFTR-dependent HCO_3^-/Cl^- transport ratio in each CF-causing CFTR mutation appears to correlate well with the reported disease severity, especially with the pancreatic status of patients (11).

An increase in intracellular cAMP by secretin is one of the major signals of pancreatic HCO_3^- secretion. Activation of the CFTR Cl^- channel and the CFTR-dependent Cl^-/HCO_3^- exchange activities was shown to be responsible for cAMP-induced HCO_3^- secretion (1, 8). It was also shown that cAMP increases electrogenic HCO_3^- permeability in the luminal membrane of guinea pig pancreas through a mechanism that has not been identified (12). In addition, intracellular calcium signaling evoked by various stimuli, such as activations of cholinergic, purinergic (P2R), and protease-activated receptors (PAR), is also known to increase fluid and HCO_3^- secretion either directly or by potentiating cAMP-mediated mechanisms (1, 13–16). Cholinergic activation through vagal efferent fibers is the major physiologic stimulus for the cephalic phase of pancreatic secretion. Recent studies have demonstrated that P2Rs control fluid and the HCO_3^- secretion of many epithelial cells including pancreatic duct cells, in an auto- or paracrine

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; P2R, purinergic receptor; PAR, protease-activated receptor; WT, wild type; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*′-tetraacetic acid; BCECF, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; m.o.i.m multiplicity of infection; PKA, cAMP-dependent protein kinase; AM, acetoxymethyl ester; MQAE, *N*-(ethoxycarbonylmethyl)-6-methoxyquinolium bromide.

manner (13, 16). Moreover, PARs, which can be activated by interstitially released trypsin during pancreatic inflammation, have been suggested to increase pancreatic secretion (14, 17). However, little is known about the precise mechanism of calcium-induced HCO_3^- and fluid secretion in pancreatic duct cells.

In the present study, we report that calcium signals activate CFTR- and Cl^--dependent HCO_3^- transport in the luminal membrane of pancreatic duct-derived cells. ATP and trypsin were found to increase the luminal Cl^-/HCO_3^- exchange activity of CAPAN-1 cells expressing wild type (WT)-CFTR, and this was inhibited by buffering $[Ca^{2+}]_i$ with 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*,*N*′-tetraacetic acid (BAPTA). The effects of ATP on luminal Cl^-/HCO_3^- exchange were not prominent in CFPAC-1 cells bearing a defective form (Δ508) of CFTR, although ATP evoked significant calcium signaling in the cells. Notably, adenoviral transfection of WT-CFTR in CFPAC-1 cells increased ATP-activated luminal Cl^-/HCO_3^- exchange. These results provide the molecular basis for calcium-induced bicarbonate secretion by pancreatic duct cells and possibly by other epithelial cells.

MATERIALS AND METHODS

Chemicals and Solutions—Fura-2-AM and 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM) were purchased from Molecular Probes (Eugene, OR). PAR2-activating peptide (SLIGRL-NH_2) and PAR4-activating peptide (AYPGKF-NH_2) were synthesized at the Korea Basic Science Institute (Seoul, Korea). All other chemicals including ATP, trypsin, and thrombin were purchased from Sigma.

The standard perfusate was termed solution A and contained (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, and 10 HEPES (pH 7.4 with NaOH). In the measurements of Cl^-/NO_3^- exchange (see Fig. 3) and $[Cl^-]_i/[HCO_3^-]_i$ exchange (see Fig. 8, *B* and *C*), Cl^- was replaced with equimolar NO_3^- or gluconate in solution A. The HCO_3^--buffered NaCl solution B contained (in mM) 120 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$ (pH 7.4 with NaOH). The HCO_3^--buffered Cl^--free solution C contained (in mM) 120 Na^+-gluconate, 5 K^+-gluconate, 1 $MgSO_4$, 9.3 hemicalcium cyclamate, 10 D-glucose, 5 HEPES, and 25 $NaHCO_3$ (pH 7.4 with NaOH). To prepare HCO_3^--buffered high K^+ (100 mM K^+) solution D, 95 mM NaCl in solution B was replaced with 95 mM KCl. To prepare HCO_3^--buffered, high K^+ (100 mM K^+), Cl^--free solution E, 95 mM Na^+ gluconate in solution C was replaced with 95 mM K^+ gluconate. All HCO_3^--buffered solutions were continuously gassed with 95% O_2 and 5% CO_2 to maintain the pH of solutions. The osmolarity of all solutions was adjusted to 310 mosM with the major salt prior to use.

Monolayer Culture and Adenoviral Transfection—CAPAN-1 (ATCC HTB-79) and CFPAC-1 cells (ATCC CRL-1918) were purchased from the American Type Culture Collection, Manassas, VA. CAPAN-1, a metastatic pancreatic cancer cell line expressing WT-CFTR, was maintained in RPMI 1640 medium (Invotrogen) supplemented with 10% fetal bovine serum and penicillin (50 units/ml)/streptomycin (50 μg/ml). CFPAC-1, a pancreatic duct cell line derived from a patient with CF bearing a ΔF508-CFTR mutation, was maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum and penicillin (50 units/ml)/streptomycin (50 μg/ml). CAPAN-1 and CFPAC-1 cells were removed from the culture flasks using trypsin/EDTA washed by centrifugation/resuspension in fresh medium. Aliquots of this suspension were plated (3×10^5 cells/cm^2) on permeable supports fabricated from Transwell-Clear Polyester membrane (0.4 μm pore diameter; Costar, Cambridge, MA), which was coated with collagen (30 μg/ml) for 30 min. Membranes bearing cultured cells (culture area 0.3 cm^2) were floated on the culture medium in Petri dishes and incubated for 4–5 days until the cells formed a functionally polarized monolayer.

Adenoviral vector expressing WT-CFTR (Ad-CFTR) was purchased from the Institute of Human Gene Therapy, Philadelphia, PA. The E1–E3 deleted viruses were amplified in HEK 293 cells and purified using a $CsCl_2$-based method (18). Ad-CFTR and Ad-β-Gal (mock vector) viruses were titrated by limiting dilutions or plaque assays and stored in small aliquots to avoid repeated freeze-thaw cycles. Filter-grown CFPAC-1 cells were washed twice with phosphate-buffered saline, and 150 μl of the virus-containing medium was applied to the luminal side of the cells for 5 h. The cells were then rinsed with fresh media and incubated in the culture medium and were used for pH_i measurements 48–72 h after transfection.

RT-PCR and Immunohistochemistry—The expression of CFTR in transfected cells was verified by RT-PCR, immunostaining, and immunoblotting using a procedure described previously (19). The mRNA transcripts of human WT-CFTR and ΔF508-CFTR were analyzed by RT-PCR in pancreatic duct-derived cells. Total RNA was prepared from CAPAN-1 and CFPAC-1 cells using Trizol solution (Invitrogen) and reverse-transcribed using random hexa-primers and an RNase H^- reverse transcriptase (Invitrogen). The cDNA was amplified using specific primers and a *Taq* polymerase (Promega, Madison, WI), and the products were separated on a 2% agarose gel containing 0.1 μg/ml ethidium bromide. The primer sequences used were as follows: WT-CFTR, sense (5′-GGCACCATTAAAGAAAATATCATCTT-3′) and antisense (5′-TAATTTGGGTAGTGTGAAGGGTTC-3′), size of PCR product 145 base pairs; ΔF508-CFTR, sense (5′-GGCACCATTAAAGAAAATATCATTGG-3′) and antisense (5′-TAATTTGGGTAGTGTGAAGGGTTC-3′), size of PCR product 142 base pairs.

Immunostaining of monolayers was performed using monoclonal antibodies against the C terminus of CFTR (Clone 24-1; R&D Systems, Minneapolis, MN). Briefly, membrane-cultured cells were fixed and permeabilized by incubation in cold methanol for 10 min at −20 °C and then stained with the primary antibodies against CFTR and the fluorescently labeled secondary antibodies. Images were collected using a Zeiss LSM510 confocal microscope with serial Z-sections. For immunoblotting, cell lysates (100 μg of protein) were suspended in SDS sample buffer and separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were then transferred to polyvinylidene difluoride membranes, which were blocked by a 1-h incubation at room temperature in 5% nonfat dry milk in a solution containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20. CFTR proteins were detected by 1-h incubations with monoclonal antibodies against the R domain of CFTR (Clone 13-1; R&D Systems) and the appropriate secondary antibodies. The staining intensities of immunostaining and immunoblotting were analyzed using an imaging software (MCID version 3.0; Brook University, St. Catharines, Ontario, Canada).

Measurement of $[Ca^{2+}]_i$ and $[Cl^-]_i$—Measurements of $[Ca^{2+}]_i$ in the monolayers were performed using protocols reported previously with slight modification (20). Briefly, after achieving confluency, the cells were loaded with Fura-2 by incubating in a medium containing 3 μM Fura-2-AM and 1.6 μM pluronic F127 (for 30 min at 37 °C). A membrane bearing Fura-2-loaded cells was mounted in a miniature Ussing chamber (AKI Institute, University of Copenhagen, Denmark) attached to the stage of an inverted microscope. The membrane was located in between the two-half chambers, which separate the chamber into a luminal (upper) and a basolateral (lower) compartment. A transparent coverslip was placed at the bottom of the perfusion chamber, which allowed fluorescence measurements from the dye-loaded monolayers using objective lenses having a long working distance (more than 2 mm). Separate luminal and basolateral perfusates were delivered to the chamber after warming (37 °C). The Fura-2 fluorescence was recorded (Delta Ram; PTI Inc., South Brunswick, NJ) at excitation wavelengths of 350 nm and 380 nm, and the 350/380 fluorescence ratio was calibrated by exposing the cells to solutions containing high and low concentrations of Ca^{2+} and 10 μM ionomycin (21).

To measure $[Cl^-]_i$, CAPAN-1 and CFPAC-1 cells were grown on coverslips, which form the bottom of a different type of chamber suitable for single perfusion. $[Cl^-]_i$ was measured with a Cl^--sensitive dye MQAE using the procedure described before with minor modifications (10). Cells were suspended in solution A containing 10 mM of MQAE and incubated for 30 min at room temperature and for 40 min at 0 °C. After chamber assembly, external MQAE was washed out by perfusing the cells with solution A. MQAE fluorescence was measured at an excitation wavelength of 350 nm and emission wavelength of 450 nm. At the end of each experiment, a calibration procedure was performed using standard Cl^--containing solutions with 150 mM K^+, 5 μM nigericin, and 10 μM tributyltin cyanide.

Measurement of pH_i—Measurements of pH_i in the monolayers were performed using a pH-sensitive fluorescent probe BCECF. Cells were loaded with BCECF by being incubated for 10 min at room temperature in solution A containing 2.5 μM BCECF-AM and mounted in the miniature Ussing chamber. BCECF fluorescence was recorded and calibrated using a protocol previously described (9). Briefly, the fluorescence at excitation wavelengths of 490 and 440 nm was recorded using the recording setup (Delta Ram; PTI Inc.) and the 490/440 ratios were calibrated intracellularly by perfusing the cells with solutions containing 145 mM KCl, 10 mM HEPES, and 5 μM nigericin with the pH adjusted to 6.2–7.6. $[Cl^-]_i/[HCO_3^-]_o$ exchange activities were estimated

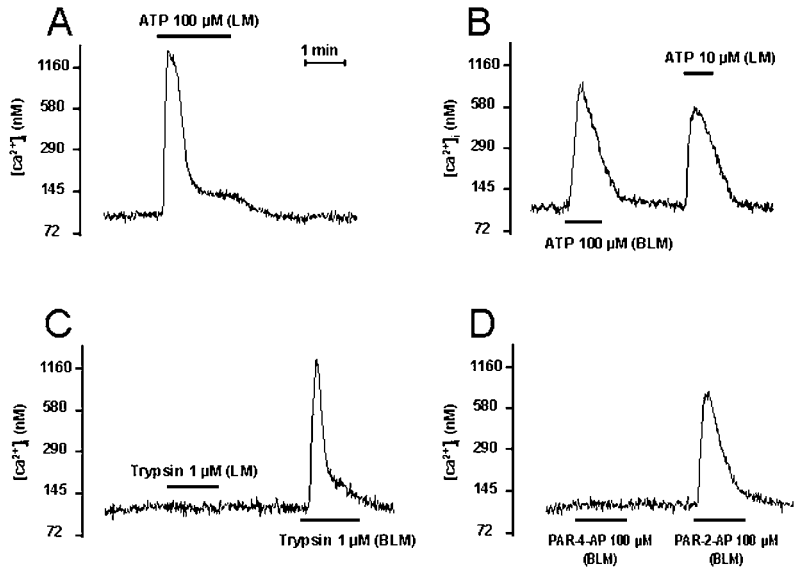

FIG. 1. **Effect of P2R and PAR agonists on $[Ca^{2+}]_i$.** Monolayers of CAPAN-1 or CFPAC-1 cells were loaded with Fura-2, and $[Ca^{2+}]_i$ was measured in separate luminal and basolateral perfusates. *A*, ATP evoked a biphasic $[Ca^{2+}]_i$ increase, a rapid peak followed by a small sustained $[Ca^{2+}]_i$ increase. *B*, both basolateral and luminal ATP increased $[Ca^{2+}]_i$, although luminal ATP showed higher affinity. Note that 10-fold lower concentration of ATP was used on the luminal side. *C*, trypsin-evoked calcium signaling only when applied to the basolateral side. *D*, among the several PAR-activating peptides (*AP*), only PAR-2-activating peptide-evoked $[Ca^{2+}]_i$ increase. CAPAN-1 and CFPAC-1 cells showed similar $[Ca^{2+}]_i$ responses to P2R or PAR agonists. *Traces A* and *D* were taken from experiments on CAPAN-1 cells, and *B* and *C* from experiments on CFPAC-1 cells. *LM*, luminal membrane; *BLM*, basolateral membrane.

from the initial rate of pH_i increase as a result of $[Cl^-]_o$ removal. Initial rates of pH_i changes were obtained from the first derivative of the traces using a single exponential fit.

Buffer capacity was calculated by measuring ΔpH_i in response to 5–40 mM NH_4Cl pulses (22). Intrinsic buffer capacity (β_i) of CAPAN-1 cells (17.6 ± 0.8 mM/pH unit at pH_i 7.2) was significantly lower than that of CFPAC-1 cells (21.6 ± 1.4 mM/pH unit). Total buffer capacity (β_t) was calculated from $\beta_t = \beta_i + 2.3[HCO_3^-]_i$ (22). At pH_i 7.2 with 5% CO_2 gassing, $[HCO_3^-]_i$ was estimated to be 14.4 mM. Thus, in HCO_3^--buffered solutions the β_t of CAPAN-1 (50.7 mM/pH unit) was only 7% different from that of CFPAC-1 (54.7 mM/pH unit). In addition, all the transporter activity comparisons were made within the same cell types. Therefore, the results of Cl^-/HCO_3^- exchange activity were expressed as pH unit/min, and this value was directly analyzed without compensating for β_t.

Statistical Analysis—The results of multiple experiments are presented as means ± S.E., and statistical analysis was carried out by using analysis of variance or Student's *t* test as appropriate. $p < 0.05$ was considered statistically significant.

RESULTS

P2Rs and PARs in Pancreatic Duct-derived Cells—To investigate the role of calcium signaling on luminal Cl^--dependent HCO_3^- transport mechanisms, we first characterized the calcium-evoking receptors in CAPAN-1 and CFPAC-1 cells (Fig. 1). Applications of ATP on either the luminal or the basolateral membrane increased $[Ca^{2+}]_i$ in both CAPAN-1 and CFPAC-1 cells, and the activation of P2Rs caused biphasic $[Ca^{2+}]_i$ increases, thus a rapid $[Ca^{2+}]_i$ peak which lasted for 30 s–1 min followed by a small sustained $[Ca^{2+}]_i$ increase (Fig. 1*A*). Repetitive short ATP applications at 3- to 5-min intervals evoked corresponding calcium signals, and during the 30 min of observation, the amplitudes of the $[Ca^{2+}]_i$ peaks did not decrease. P2Rs on the luminal membrane showed a higher affinity for ATP than those on the basolateral membrane (Fig. 1*B*). The EC_{50} values of luminal and basolateral ATP for $[Ca^{2+}]_i$ increase in CAPAN-1 cells were 3.1 μM and 20.6 μM, respectively. CFPAC-1 cells showed similar ATP responses. Thus, CFPAC-1 cells responded to ATP applications with higher affinity for luminal side (EC_{50} = 6.1 μM) than basolateral (EC_{50} = 19.9 μM). These results are in good agreement with those of our previous reports that native submandibular and pancreatic ducts express multiple P2Rs in both luminal and basolateral membranes (13, 21).

Increasing evidence suggests that pancreatic duct cells express PARs and that this plays significant roles in both physiologic and pathological states (14, 17, 23). Trypsin (1 μM) evoked a rapid Ca^{2+} peak when applied to the basolateral side of CAPAN-1 and CFPAC-1 cells, but not to the luminal side (Fig. 1*C*). Basolateral applications of isoform-specific activating peptide (AP) showed that pancreatic duct cells express functional PAR-2 receptors (Fig. 1*D*). Unlike purinergic activation, repetitive applications of PAR agonists did not cause any calcium signaling due to receptor internalization and degradation, which is a characteristic of all known PARs (23). After a resting period of 30 min, the cells began to respond to PAR agonists, possibly because of new receptor expression on the plasma membrane (data not shown).

P2R and PAR Activate Electroneutral Cl^-/HCO_3^- Exchange on the Luminal Membrane—Initially, we examined the effects of purinergic and protease-activated receptors on the Cl^--dependent HCO_3^- transport mechanisms in physiologic buffers, which contained 5 mM K^+,129 mM Cl^-, and 25 mM HCO_3^- (gassing with 5% CO_2). The basal pH_i of CFPAC-1 cells (7.23 ± 0.05) seemed to be higher than that of CAPAN-1 cells (7.11 ± 0.06). To better observe lumen-specific Cl^-/HCO_3^- exchange activity, the basolateral side of the monolayer was first applied to Cl^--free solutions. In a previous paper, we showed that this maneuver prevents the dissipation of luminally accumulated HCO_3^- through the forward mode of basolateral Cl^-/HCO_3^- exchange (outward HCO_3^- movement) (10). Although basolateral Cl^--free solution may activate the reverse mode of basolateral Cl^-/HCO_3^- exchange (inward HCO_3^- movement), intracellular alkalinization was very small; ΔpH_i increases in CAPAN-1 and CFPAC-1 cells were 0.05 ± 0.02 and 0.04 ± 0.03, respectively. This was attributed to the higher luminal to basolateral antiporter distribution ratio of the pancreatic duct cells (8, 24).

Luminal Cl^--dependent HCO_3^- transport was estimated by measuring the initial pH_i increase due to $[Cl^-]_i/[HCO_3^-]_o$ exchange. In physiologic buffers, the activation of P2R or PAR

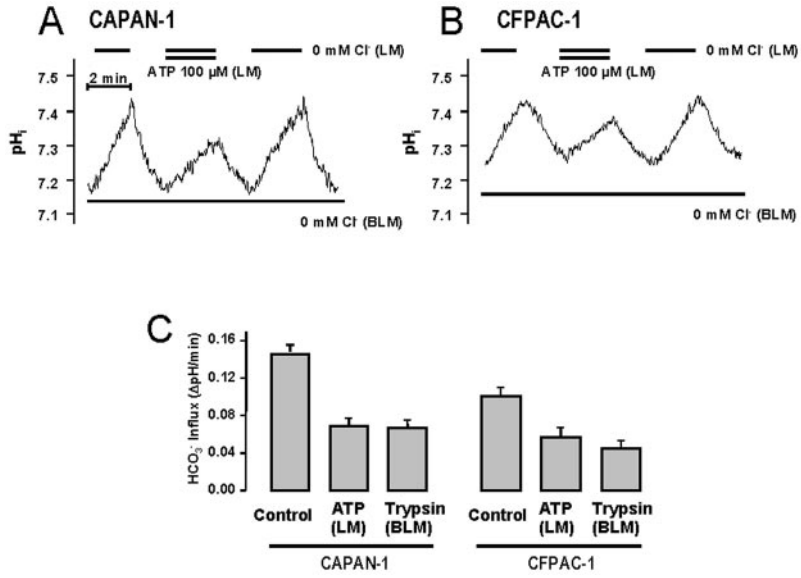

FIG. 2. **Measurements of luminal Cl^-/HCO_3^- exchange in physiologic buffers.** *A and B*, monolayers of CAPAN-1 and CFPAC-1 cells were loaded with BCECF, and pH_i was measured in a double perfusion chamber. In physiologic buffers containing 5 mM K^+, activation of either P2R or PAR decreased the observed luminal Cl^-/HCO_3^- exchange. *C*, results shown are the means ± S.E. of five experiments.

decreased the observed luminal Cl^-/HCO_3^- exchange in both CAPAN-1 and CFPAC-1 cells (Fig. 2). A similar phenomenon was observed previously in isolated submandibular duct cells and microperfused pancreatic duct cells, when they were activated by cAMP (10). We reason that this is because of the simultaneous activation of Cl^- exit pathways, such as Cl^- channels or K^+-Cl^- cotransport. Because $[Cl^-]_i$ decreases very rapidly due to the activation of Cl^- exit pathways, it attenuates the driving force for $[Cl^-]_i/[HCO_3^-]_o$ exchanges. Actually, it has been shown that both CAPAN-1 and CFPAC-1 cells express Ca^{2+}-activated Cl^- channels on their luminal membranes (25).

Therefore, in the next set of experiments we measured electroneutral luminal Cl^-/HCO_3^- exchange in high $[K^+]_o$ conditions. When countercurrent K^+ movement is blocked by high K^+ (100 mM) buffers, electrogenic Cl^- pathways are impaired, and thus most of the Cl^- movement is the result of electroneutral transport (see Fig. 4*A*). In addition, high external K^+ also blocks outward Cl^- movement through K^+-Cl^- cotransport. This was recently identified in the basolateral membrane of rat pancreatic duct cells (26). Moreover, it was found that bathing the cells in high K^+-containing buffers reduces Cl^-/NO_3^- exchange in pancreatic duct-derived cells (Fig. 3). It has previously been shown that the measurement of Cl^-/NO_3^- exchange in HCO_3^--free buffer (nominally Hepes-buffered) accurately reports Cl^- channel activity in intact cells (11). Changes in $[Cl^-]_i$ were measured by applying Cl^--free, NO_3^--containing perfusates in coverslip grown cells. Applications of 0 mM Cl^-, 145 mM NO_3^- solution to CAPAN-1 cells rapidly decreased $[Cl^-]_i$, and ATP stimulation further enhanced this $[Cl^-]_i$ decrease by 43% (Fig. 3*A*). As shown in Fig. 3*B*, perfusing the cells with a 100 mM K^+-containing solution inhibited Cl^-/NO_3^- exchange activity in control and ATP-stimulated states by 71 and 73%, respectively. Measurements in CFPAC-1 cells showed similar results and summarized results are presented in Fig. 3*C*.

Surprisingly, when measured in high-K^+ containing buffers, the activation of P2R or PAR highly increased luminal Cl^--dependent HCO_3^- transport in CAPAN-1 cells (Fig. 4, *C* and *D*). An average of 5–6 experiments are summarized in Fig. 4*B*. The basal activity of luminal Cl^-/HCO_3^- exchange was 0.145 ± 0.010 pH unit/min, which increased to 0.383 ± 0.044 and 0.481 ± 0.052 by ATP and trypsin treatment, respectively. On the other hand, the effect of calcium agonists on luminal Cl^-/HCO_3^- exchange was very small in CFPAC-1 cells. As shown in Fig. 4, *E* and *F*, CFPAC-1 cells showed only 27% increase in Cl^-/HCO_3^- exchange activity when treated with ATP *versus* the corresponding 164% activity increase in CAPAN-1 cells (Fig. 4*B*).

Expression of WT-CFTR Augments the Effect of Calcium Agonists on Luminal Cl^-/HCO_3^- Exchange in CFTR-impaired Cells—The finding that the effect of calcium agonists is nearly abolished in CFTR-impaired cells prompted us to investigate the role of CFTR. Accordingly, we measured luminal Cl^-/HCO_3^- exchange activity in membrane-cultured CFPAC-1 cells 48–72 h after transfection with variable doses of Ad-CFTR. The expression of WT-CFTR in CFPAC-1 monolayer was verified by several molecular methods (Fig. 5).

The presence of the mRNA transcripts of WT- and ΔF508-CFTR in RT-PCR of CAPAN-1 and CFPAC-1 cells, respectively, confirmed the cell-line characteristics. As expected, both WT- and ΔF508-CFTR transcripts were found in CFPAC-1 cells transfected with Ad-CFTR (Fig. 5*A*). By immunostaining, when the cells were transfected with 30 and 200 m.o.i. (multiplicity of infection) of Ad-CFTR, 31 ± 7% and 83 ± 6% of the cells, respectively, were found to be CFTR-positive. When the amount of CFTR protein was compared by immunoblotting, the CFTR expression in CFPAC-1 cells transfected with 200 m.o.i. of Ad-CFTR was 2.7-fold that of CAPAN-1 cells (Fig. 5*B*). On the other hand, when the same comparison was made by the luminal surface immunostaining using semi-quantitative confocal microscopy, the luminal membrane CFTR expression in 200 m.o.i. transfected CFPAC-1 cells was 1.4-fold that of CAPAN-1 cells (Fig. 5*C*). Several possible mechanisms can explain this discrepancy between the results obtained by immunoblotting and surface confocal microscopy, including the possibility of higher protein maturation in the native expression of CAPAN-1 cells. However, this mechanism was not further investigated in the present study.

The expression of CFTR significantly enhanced the effect of ATP on luminal Cl^-/HCO_3^- exchange in a dose-dependent manner. In non-transfected and Ad-β-Gal (mock vector)-transfected cells, ATP increased luminal Cl^-/HCO_3^- exchange activity by 27 and 24%, respectively. However, in the exogenously CFTR-expressed cells by transfecting with 30 and 200 m.o.i. of Ad-CFTR, ATP increased luminal Cl^-/HCO_3^- exchange activity by 60 and 246%, respectively (Fig. 6).

Calcium Is Required for the P2R- or the PAR-induced Activation of Luminal Cl^-/HCO_3^- Exchange—As depicted in Fig. 1, stimulation by P2R or PAR caused a rapid $[Ca^{2+}]_i$ rise and a short-lived $[Ca^{2+}]_i$ peak in pancreatic duct-derived cells. Interestingly, treatment with P2R or PAR agonists 2–3 min before

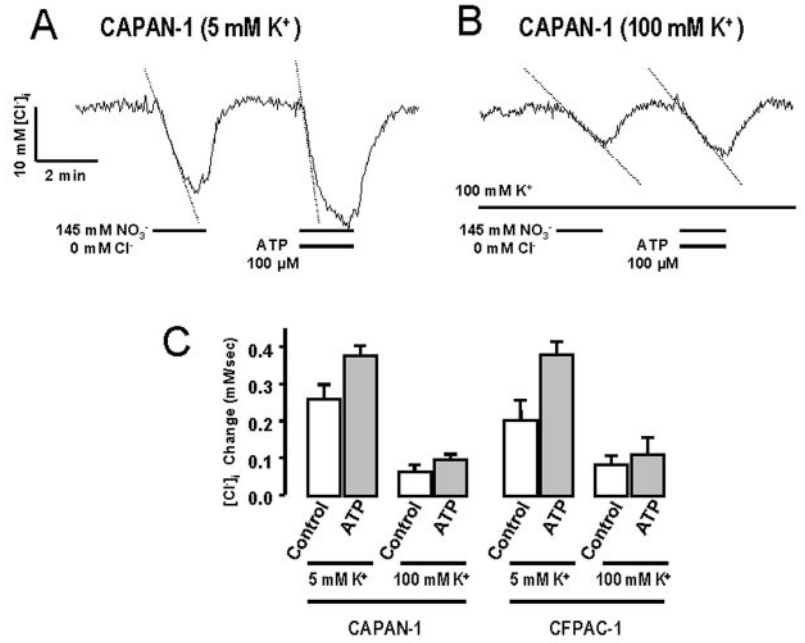

FIG. 3. **Measurements of Cl^-/NO_3^- exchange.** Cl^-/NO_3^- exchange was measured in HCO_3^--free buffer (nominally Hepes-buffered), which reports Cl^- channel activity in intact cells. $[Cl^-]_i$ was measured with the aid of the Cl^--sensitive dye MQAE in coverslip-grown CAPAN-1 and CFPAC-1 cells. *A*, applications of NO_3^- solution to CAPAN-1 cells rapidly decreased $[Cl^-]_i$, and ATP further enhanced the $[Cl^-]_i$ decrease. *B*, perfusing the cells with a high K^+-containing solution inhibited Cl^-/NO_3^- exchange activities. *C*, the results shown are the means ± S.E. of four to six experiments.

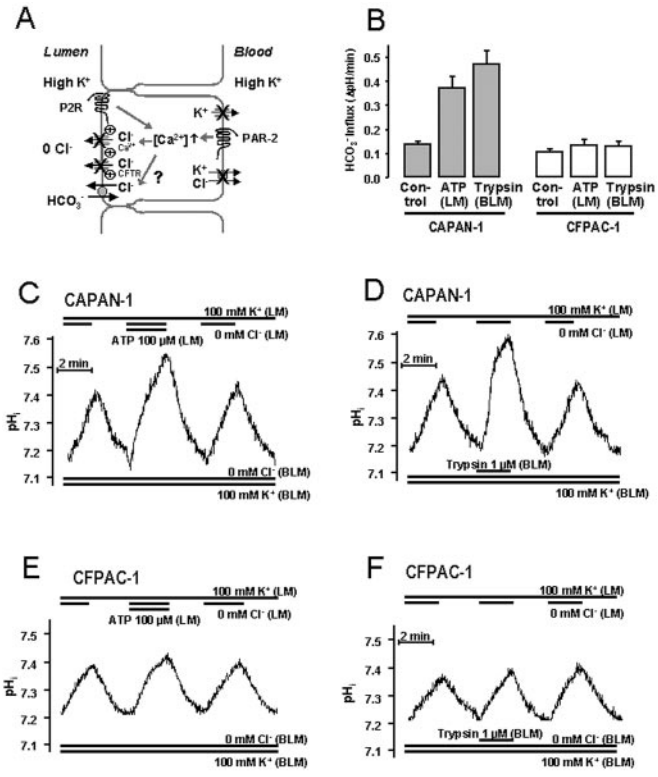

FIG. 4. **Measurements of luminal Cl^-/HCO_3^- exchange in high K^+ buffers.** The activities of luminal Cl^-/HCO_3^- were measured in perfusate containing a high concentration of K^+ (100 mM) to block Cl^- movement through electrogenic or K^+-coupled pathways. *A*, schematic diagram of Cl^- and HCO_3^- movement in high K^+ buffer. *B*, results shown are the means ± S.E. of five to six experiments. Activation of P2R- (*C*) or PAR-stimulated (*D*) luminal Cl^-/HCO_3^- exchange in CAPAN-1 cells, but not in CFPAC-1 cells (*E* and *F*).

the application of Cl^--free solution did not increase the luminal Cl^-/HCO_3^- exchange (Fig. 7*A*), although treatment with agonists simultaneously with or 15 s after Cl^--free solution strongly activated CFTR-dependent luminal Cl^-/HCO_3^- exchange (Fig. 4). These results suggest that the Cl^--dependent HCO_3^- transport activity is highly correlated with $[Ca^{2+}]_i$. To investigate the Ca^{2+}-dependence of the P2R- or the PAR-induced effects, CAPAN-1 cells were preloaded with BAPTA-AM (50 μM) for 30 min and luminal Cl^-/HCO_3^- exchange activities were measured after ATP or trypsin stimulation. Chelating intracellular calcium with BAPTA completely abolished the P2R- or PAR-induced activation of luminal Cl^-/HCO_3^- exchange (Fig. 7, *B* and *D*).

It has been suggested that Gq-coupled agonists may activate CFTR Cl^- channel function by increasing responsiveness to endogenous cAMP/PKA pathways (27). To determine whether P2R or PAR activates CFTR-dependent Cl^-/HCO_3^- exchange via the PKA pathway, cells were pretreated with a high concentration (30 μM) of the PKA inhibitor H89 for 1 h, and the activity of Cl^-/HCO_3^- exchange was measured with buffers containing 50 nM H89. As shown in Fig. 7, *C* and *D*, the inhibition of the PKA pathway did not decrease P2R- or PAR-induced activation of luminal Cl^-/HCO_3^- exchange. The PKA-inhibitory effect of H89 was confirmed by the finding that the same H89 treatment inhibited the forskolin-induced activation of luminal Cl^-/HCO_3^- exchange by 93% in CAPAN-1 cells.

Measurements of Cl^-/OH^- Exchange and $[Cl^-]_o/[HCO_3^-]_i$ Exchange—Finally, we demonstrated that the increases in pH_i by $[Cl^-]_o$ removal in the present study were caused by a Cl^-/HCO_3^- exchanger, although other mechanisms, for example, Cl^-/OH^- exchange or the exchange of Cl^- and HCO_3^- via Cl^- channels may also have a similar effect. First, the activity of Cl^-/OH^- exchange was measured in CAPAN-1 cells using HCO_3^-- and CO_2-free solutions (Hepes-buffered). As shown in Fig. 8*A*, Cl^-/OH^- exchange activity was not observed in the luminal membrane of CAPAN-1 cells. Applications of Cl^--free solution caused a slight decrease or no changes in pH_i, and ATP treatment did not evoke a pH_i increase in Hepes-buffered solutions. Hence, the activity of Cl^-/OH^- cannot explain the increases in pH_i by $[Cl^-]_o$ removal in HCO_3^-- and CO_2-containing solutions (Fig. 4).

It has been shown that HCO_3^- can pass through several Cl^- channels including CFTR and the Ca^{2+}-activated Cl^- channel, although its permeability is much lower than that of Cl^- (8, 12). It is possible that depolarization caused by Cl^- exit may evoke the inward flux of HCO_3^- through Cl^- channels during the reverse mode measurements of Cl^-/HCO_3^- exchange ($[Cl^-]_i/[HCO_3^-]_o$ exchange, Fig. 4). However, the fact that bath-

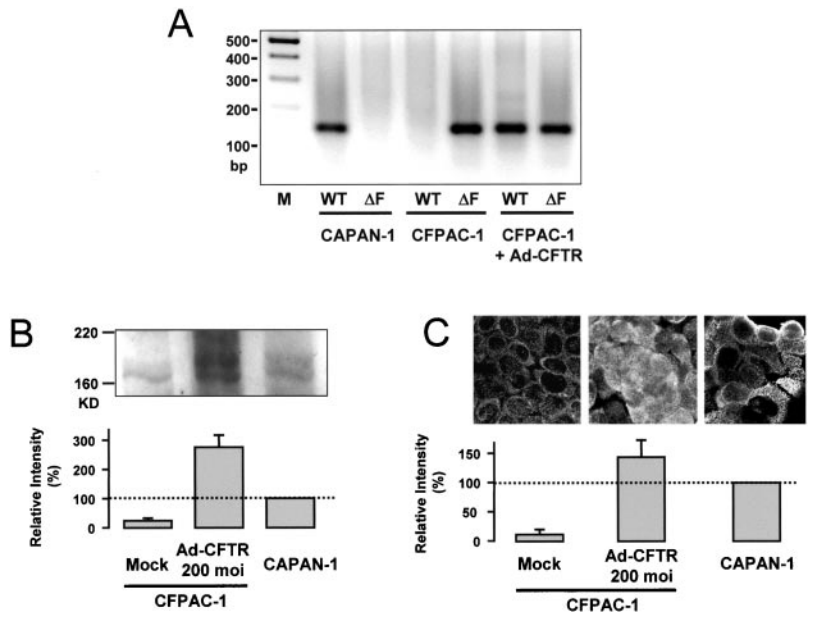

FIG. 5. **Exogenous expression of WT-CFTR in CFPAC-1 cells by Ad-CFTR transfection.** *A*, RT-PCR. mRNA transcripts of WT- and ΔF508-CFTR were analyzed in pancreatic duct-derived cells. Both WT- and ΔF508-CFTR transcripts were detected in Ad-CFTR transfected CFPAC-1 cells. *B*, immunoblotting of CFTR protein was performed 48 h after Ad-CFTR transfection using monoclonal antibodies against the R-domain of CFTR. *C*, semi-quantitative confocal microscopy on luminal membrane CFTR expression. Monolayers of pancreatic duct-derived cells were labeled with antibodies against the C terminus of CFTR and with fluorescently tagged secondary antibodies. Under the same image-acquisition setup for each sample, luminal surface images were collected using a Zeiss LSM510 microscope after serial Z-axis sections. Summarized results are the averages of three separate experiments.

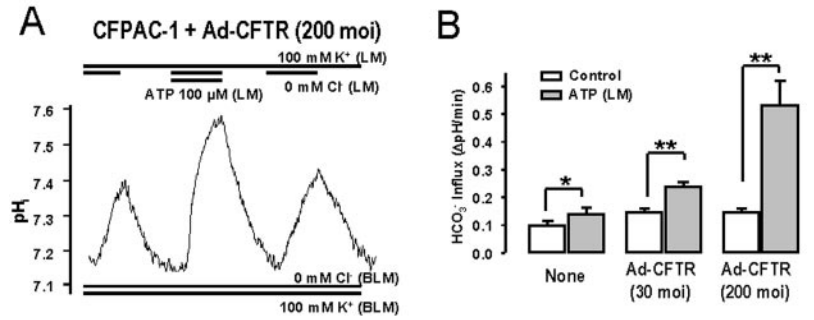

FIG. 6. **WT-CFTR expression augments the effect of calcium agonists on luminal Cl^-/HCO_3^- exchange in CFPAC-1 cells.** CFPAC-1 cells were transfected with various doses of Ad-CFTR, and luminal Cl^-/HCO_3^- exchange was measured using the methods described in Fig. 3. Transfection of Ad-CFTR increased the stimulatory effect of ATP on luminal Cl^-/HCO_3^- exchange (*A*). The averages of five experiments are shown in *panel B*.

ing the cells in 100 mM K^+ decreased Cl^- channel activity (Fig. 3) but enhanced the pH_i increase by $[Cl^-]_o$ removal in ATP-stimulated states (compare Fig. 2 with Fig. 4) strongly suggests that HCO_3^- movement through Cl^- channels cannot explain the present finding. In addition, we reevaluated the effect of electrogenic HCO_3^- pathways using the forward mode of Cl^-/HCO_3^- exchange ($[Cl^-]_o/[HCO_3^-]_i$ exchange, Fig. 8*B*). When we changed the perfusates from HCO_3^-- and CO_2-containing solutions to Hepes-buffered solutions, pH_i abruptly increased due to the rapid diffusion of CO_2. Cells can regain optimum pH_i by two mechanisms, via the forward mode of $[Cl^-]_o/[HCO_3^-]_i$ exchange and alternatively via the electrogenic HCO_3^- exit through Cl^- channels. In this state, the directions of Cl^- and HCO_3^- movement through Cl^- channels are both outward. Therefore, the exchange of Cl^- and HCO_3^- through anion channels cannot occur, and the HCO_3^- movement through Cl^- channels can be isolated from the activity of Cl^-/HCO_3^- exchange by measuring the pH_i recovery in Cl^--free solutions. Because CO_2 can nonspecifically diffuse through both luminal and basolateral membranes, coverslip-grown cells were used for the following experiments. When applying the Hepes-buffered solution, cells were stimulated with ATP to fully activate Cl^- channels. The pH_i recovery rate of CAPAN-1 cells from alkali load was 0.246 ± 0.025 pH unit/min (at pH_i 7.6) in Cl^--containing solutions. However, in Cl^--free solutions this value dropped to 0.041 ± 0.014 pH unit/min. These results imply that HCO_3^- movement through Cl^- channels mediates less than 17% of the total HCO_3^- transport and that this mechanism performs only a minor role in CAPAN-1 cells (Fig. 8*B*).

We also verified the stimulatory effect of ATP on the forward mode of $[Cl^-]_o/[HCO_3^-]_i$ exchange (Fig. 8*C*). Experiments were also performed in high K^+-containing solutions to minimize the effect of electrogenic pathways. In 100 mM K^+-containing solution, the basal pH_i recovery rate was 0.081 ± 0.009 pH unit/min (at pH_i 7.6) and ATP stimulation increased the pH_i recovery rate by 72% (0.140 ± 0.009 pH unit/min, at pH_i 7.6). Comparing to 164% increase by ATP in the reverse mode of $[Cl^-]_o/[HCO_3^-]_i$ exchange (Fig. 4*B*), the increase rate was smaller in the forward mode of $[Cl^-]_o/[HCO_3^-]_i$ exchange (Fig. 8*C*). This was possibly due to the progressive increase in $[Cl^-]_i$ by the blockade of Cl^- exit pathways in high K^+-containing solutions.

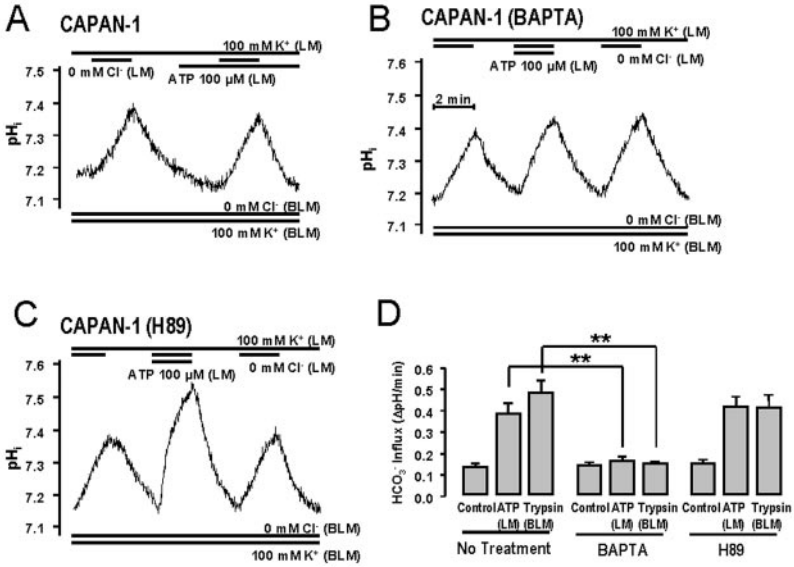

FIG. 7. **Calcium is required for the P2R- or the PAR-induced activation of luminal Cl^-/HCO_3^- exchange.** *A*, application of ATP 2 min before Cl^--free solution did not increase luminal Cl^-/HCO_3^- exchange. *B* and *C*, effect of ATP on luminal Cl^-/HCO_3^- exchange was measured in CAPAN-1 cells after pretreatment with the calcium chelator BAPTA-AM (*B*, 50 μM for 30 min) or the PKA inhibitor H89 (*C*, 30 μM for 1 h). BAPTA pretreatment completely abolished the stimulatory effect of ATP on luminal Cl^-/HCO_3^- exchange, whereas H89 pretreatment did not. *D*, summary data of calcium chelator (*B*) and PKA inhibitor (*C*) pretreatments. Means ± S.E. of five to six individual traces of P2R or PAR activation are shown. **, $p < 0.01$

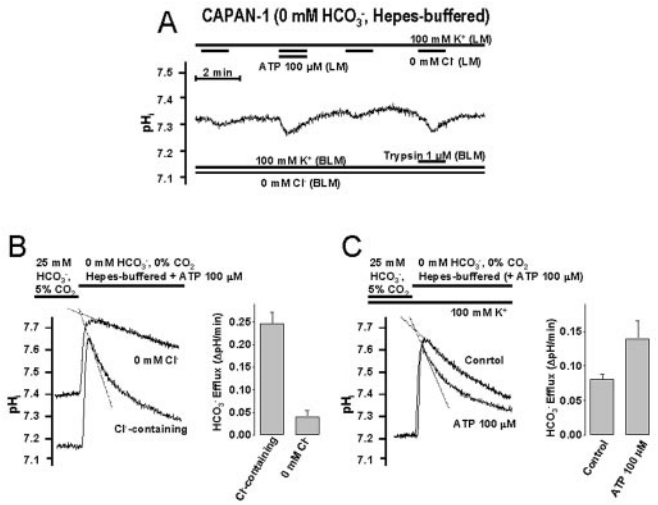

FIG. 8. **Measurements of Cl^-/OH^- exchange and $[Cl^-]_o/[HCO_3^-]_i$ exchange.** *A*, Cl^-/OH^- exchange was measured in CAPAN-1 cells using HCO_3^-- and CO_2-free solutions (Hepes-buffered). Cl^-/OH^- exchange activities were not observed in the luminal membrane of CAPAN-1 cells. Five separate experiments showed similar results. *B*, to evaluate the effect of electrogenic HCO_3^- pathways, the forward mode of Cl^-/HCO_3^- exchange ($[Cl^-]_o/[HCO_3^-]_i$ exchange) was measured in Cl^--containing and Cl^--free solutions. *C*, stimulatory effect of ATP on the forward mode of $[Cl^-]_o/[HCO_3^-]_i$ exchange. Summarized results are the means ± S.E. of five to six experiments.

DISCUSSION

The coordinated actions of various transporters on both the luminal and basolateral membranes of pancreatic duct cells perform transepithelial HCO_3^- transport. Although the precise mechanisms of how duct cells can secrete such high concentrations of HCO_3^- (up to 140 mM) are unknown, the following two processes are generally accepted as important pathways of ductal HCO_3^- secretion (8). First, CFTR performs a critical role in HCO_3^- secretion. This is supported by the observation that HCO_3^- secretion is severely defective in the pancreatic juice of CF patients (2, 3). Second, Cl^-/HCO_3^- exchange at the luminal membrane mediates significant portions of HCO_3^- secretion and accounts for the transport of at least 70–80 mM concentrations of HCO_3^- in the pancreatic juice (8). Recently, we demonstrated that the activity of luminal Cl^-/HCO_3^- exchange depends on CFTR expression (9, 10). A series of studies support the notion that CFTR-dependent pancreatic HCO_3^- secretion is critical for maintaining the patency of ductal trees (2, 3, 11). Thus, the impairment of this process was suggested to be an important pathologic mechanism in mutated CFTR-related pancreatic damage and possibly other organ pathologies (4).

It has long been known that calcium agonists such as cholecystokinin or acetylcholine can increase fluid and HCO_3^- secretion and that these have a strong potentiatory effect when combined with cAMP agonists, such as secretin or vasoactive intestinal polypeptide (VIP), in *in vivo* experiments (1, 15). However, the molecular mechanisms responsible for Ca^{2+}-induced fluid and HCO_3^- secretion are largely unknown. Recently, it was suggested that the activation of the Ca^{2+}-activated Cl^- channel by P2R activation in pancreatic duct cells can stimulate HCO_3^- secretion by the parallel activation of electroneutral Cl^-/HCO_3^- exchange, as a substitute for CFTR Cl^- channel (25). However, this study focused on electrogenic Cl^- conductance, not on electroneutral Cl^-/HCO_3^- exchange, the transporter that actually mediates HCO_3^- transport. Moreover, the study was undertaken in nonpolarized cultures, and thus it is impossible to isolate the luminal membrane-specific effect.

Our results demonstrate that calcium agonists activate CFTR- and Cl^--dependent HCO_3^- transport in the luminal membrane of pancreatic duct cells. 1) Activation of P2R or PAR stimulated luminal Cl^-/HCO_3^- exchange only in CAPAN-1 cells expressing WT-CFTR, but not in CFPAC-1 cells bearing an impaired CFTR, although the calcium agonists evoked similar $[Ca^{2+}]_i$ elevation in both cells (Figs. 1 and 4). 2) The stimulatory effects of calcium agonists on luminal Cl^-/HCO_3^- exchange were well correlated with the duration of high $[Ca^{2+}]_i$ peak, and these effects were completely abolished by BAPTA pretreatment (Fig. 7). Although other Gq-coupled events such as the activation of PKC (27) or the direct stimulation by heterotrimeric G proteins (28) cannot be completely excluded as a possible stimulatory mechanism, the above findings suggest that $[Ca^{2+}]_i$ increase is a prerequisite for the P2R- or PAR-mediated stimulation of luminal Cl^-/HCO_3^- exchange. 3) Notably, exogenous CFTR expression by Ad-CFTR transfection substantially increased the stimulatory effect of calcium agonists on luminal Cl^-/HCO_3^- exchange in CFPAC-1 cells (Fig. 6).

Although cAMP is regarded as the major intracellular signal for HCO_3^- secretion, agonists that evoke calcium signals also stimulate HCO_3^- secretion in many CFTR-expressing epithelia. Therefore, it is not surprising that not only cAMP-activated but

also Ca^{2+}-activated HCO_3^- secretion was found to be impaired in the intestinal epithelia of CFTR(−/−) mice (29, 30). In the present study, purinergic stimulation increased luminal Cl^-/HCO_3^- exchange only by 27% in CFTR-impaired CFPAC-1 cells. However, this value jumped to 164 and 246% in endogenous (CAPAN-1, Fig. 4*B*) and exogenous CFTR-expressing cells (CFPAC-1 transfected with 200 m.o.i. of Ad-CFTR, Fig. 6), respectively. These findings are comparable with the data obtained from mouse duodenum in which cholinergic stimulation increased HCO_3^- secretion by 168% in CFTR(+/+) mice but only by 67% in CFTR(−/−) mice (30). When combined, these results suggest that CFTR plays an important role in the epithelial HCO_3^- secretion mediated by various intracellular signals. Therefore, we expect that without the restoration of CFTR expression in the luminal membrane, the application of calcium agonists such as ATP may regain only a limited amount of HCO_3^- secretion in CF epithelia, if any.

The present findings may be applicable to other CFTR-expressing epithelia. For example, the primary sweat from CF patients is acidic and hypertonic, suggesting that there is some defect in HCO_3^--driven fluid secretion (31, 32). Interestingly, the physiologic stimuli for sweat secretion, both sympathetic and parasympathetic, are mediated by calcium-coupled cholinergic receptors. Although it was originally hinted from studies upon sweat glands that the disease-related protein, which we call now CFTR, is a cAMP-activated Cl^- channel (33, 34), the cAMP signal is not the major physiologic signal in human sweat glands (35). According to the present study, a defect in Ca^{2+}-activated HCO_3^- transport, which depends on CFTR expression, can be used to explain the puzzling abnormalities of CF sweat.

In addition to the classical cholinergic and cholecystokinin pathways, recent studies have revealed the importance of other calcium-related receptors, such as P2R and PAR, in the regulation of pancreatic fluid and HCO_3^- secretion. An interesting suggestion is that cholinergic stimulation releases ATP from zymogen granules in acinar cells, and that this in turn activates P2Rs in the luminal membrane of duct cells (16). Combining the present data of P2R-induced HCO_3^- secretion with the above idea provides a possible molecular basis for the strong potentiation of pancreatic fluid secretion by cholinergic stimulations *in vivo*.

The activation of PARs, which may be prominent during pancreatic inflammation due to proteolytic cleavage by tryptase released from mast cells or autoactivated trypsin from acinar cells (23), evokes a complicated pattern of pancreatic juice secretion, characterized by a prompt increase followed by a transient suppression and a subsequent protracted increase (14). Observations made in the present study may provide explanations for this complicated pattern of secretion. Initial PAR activation caused a single calcium peak, but subsequent application of agonists did not evoke calcium signaling due to receptor internalization and degradation. Cells began to regain their response to PAR agonists 30 min after initial stimulation caused by new receptor expression on the membrane (see Fig. 1 and the relevant result section). Therefore, the secretory activity by PAR stimulation closely follows $[Ca^{2+}]_i$ increase and subsequent Cl^-/HCO_3^- exchange activity.

In conclusion, the regulation of HCO_3^- transport by calcium signaling demonstrated here may be of particular significance for the understanding of the physiologic and pathologic roles of calcium-mediated bicarbonate secretion. Activation of CFTR- and Cl^--dependent HCO_3^- transport provide the molecular basis for pancreatic fluid and bicarbonate secretion by calcium agonists. In addition, impairments in the above mechanism can explain the puzzling defect of calcium-induced HCO_3^- secretion in several CF epithelia including intestinal mucosa and sweat glands, which, in turn, highlights the general importance of CFTR in epithelial bicarbonate secretion induced by various stimuli.

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