Ca^{2+}-activated Cl^- channels can substitute for CFTR in stimulation of pancreatic duct bicarbonate secretion[1]

ÁKOS ZSEMBERY,*,†,‡ MARIO STRAZZABOSCO,† AND JÜRG GRAF*,[2]

*Department of General and Experimental Pathology, University of Vienna, Vienna, Austria; †Istituto di Medicina Interna, Università ed Azienda Ospedaliera di Padova, Padua, Italy; and ‡Department of Pathophysiology, Semmelweis University, Faculty of Medicine, Budapest, Hungary

ABSTRACT **This study addresses the mechanisms by which a defect in CFTR impairs pancreatic duct bicarbonate secretion in cystic fibrosis. We used control (PANC-1) and CFTR-deficient (CFPAC-1; ΔF508 mutation) cell lines and measured HCO_3^- extrusion by the rate of recovery of intracellular pH after an alkaline load and recorded whole cell membrane currents using patch clamp techniques. *1)* In PANC-1 cells, cAMP causes parallel activation of Cl^- channels and of HCO_3^- extrusion by DIDS-sensitive and Na^+-independent Cl^-/HCO_3^- exchange, both effects being inhibited by Cl^- channel blockers NPPB and glibenclamide. *2)* In CFPAC-1 cells, cAMP fails to stimulate Cl^-/HCO_3^- exchange and Cl^- channels, except after promoting surface expression of ΔF508-CFTR by glycerol treatment. Instead, raising intracellular Ca^{2+} concentration to 1 μmol/l or stimulating purinergic receptors with ATP (10 and 100 μmol/l) leads to parallel activation of Cl^- channels and HCO_3^- extrusion. *3)* K^+ channel function is required for coupling cAMP- and Ca^{2+}-dependent Cl^- channel activation to effective stimulation of Cl^-/HCO_3^- exchange in control and CF cells, respectively. It is concluded that stimulation of pancreatic duct bicarbonate secretion via Cl^-/HCO_3^- exchange is directly correlated to activation of apical membrane Cl^- channels. Reduced bicarbonate secretion in cystic fibrosis results from defective cAMP-activated Cl^- channels. This defect is partially compensated for by an increased sensitivity of CF cells to purinergic stimulation and by alternative activation of Ca^{2+}-dependent Cl^- channels, mechanisms of interest with respect to possible treatment of cystic fibrosis and of related chronic pancreatic diseases.—Zsembery, Á., Strazzabosco, M., Graf, J. Ca^{2+}-activated Cl^- channels can substitute for CFTR in stimulation of pancreatic duct bicarbonate secretion. *FASEB J.* 14, 2345–2356 (2000)**

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CYSTIC FIBROSIS (CF) is the most common hereditary disorder among the Caucasian population (1, 2). The disease affects cAMP-dependent electrolyte transport in a variety of organs resulting, as the most prominent manifestations of the disease, in progressive respiratory and pancreatic insufficiency. In the pancreas, obstruction of the ductal system is followed by cystic dilatation, fibrosis and atrophy of the gland (3). CF is caused by mutations in the gene that encodes for CFTR, the cystic fibrosis transmembrane conductance regulator (4, 5). Of more than 800 known mutations, the majority of patients (~70%) present with the homozygous ΔF508 deletion. Furthermore, heterozygosis in the CFTR defect has been identified as a risk factor for chronic pancreatitis (6).

CFTR functions mainly as a low conductance cAMP/PKA-activated epithelial Cl^- channel (7, 8), but it may participate in trafficking of certain proteins (9) and in the regulation of other membrane transport mechanisms such as sodium- (ENaC) (10, 11), potassium- (12, 13) and cAMP-independent outwardly rectifying chloride channels (ORCC) (14, 15), water transport (16), and cellular secretion of ATP (15, 17), which could lead to autocrine activation of purinergic receptors. Furthermore, intracellular vesicle acidification (18) and regulation of intracellular pH (pH_i), including the activity of chloride/bicarbonate exchange, appear to be under the control of CFTR (19–21). However, the mechanistic links between CFTR and these ion transporters remain to be elucidated.

CFTR assumes different functions in various organs. In the pancreas, CFTR is expressed at high levels in the apical plasma membrane of duct cells (22, 23). In these cells, secretin promotes secretion of HCO_3^- via a mechanism that appears to involve three consecutive steps: *1)* activation of the adenylate cyclase signal transduction pathway, *2)* activation of basolateral K^+ and apical Cl^- channels, and *3)* stimulation of apical Cl^-/HCO_3^- exchange that is driven by both a low intracellular Cl^- concentration

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[2] Correspondence: Department of General and Experimental Pathology, University of Vienna, Währinger Gürtel 18–20, 1090 Vienna, Austria. E-mail: juerg.graf@univie.ac.at

($[Cl^-]_i$) and high $[HCO_3^-]_i$ (24, 25). Pancreatic duct HCO_3^- secretion is impaired in the course of CF (26, 27), suggesting that CFTR is the Cl^- channel that cooperates with Cl^-/HCO_3^- exchange to promote secretin/cAMP-dependent HCO_3^- secretion. Intracellular cAMP is also generated by other hormones [VIP (28), PHI (29), β-adrenergic agonists (30)] that may activate CFTR. In addition, measurements of short circuit current indicate that other agonists promote anion transport in pancreatic duct cells via Ca^{2+} signaling [purinergic agonists (31–33), cholinergic (34), angiotensin II (35), and histamine (36)]. However, it is unknown whether this alternative signal transduction pathway may sustain fluid and HCO_3^- secretion in CFTR-deficient cells and could thus be exploited for ameliorating the course of the disease in some patients affected by CF (37).

The aim of this study was to directly correlate cAMP- and Ca^{2+}-dependent activation of Cl^- channels with Cl^-/HCO_3^- exchange activity in control and CFTR-deficient pancreatic duct cells. We used pancreatic duct cell lines derived from control and CFTR-deficient (ΔF508/ΔF508) ductal adenocarcinomas, PANC-1 and CFPAC-1, respectively (38, 39). The ΔF508/ΔF508 deletion present in CFPAC-1 cells belongs to a group of CFTR mutations that result in impaired targeting to the plasma membrane of the otherwise functional protein (40, 41). This defect can be restored by exposing the cells to chemical chaperons such as glycerol, resulting in proper expression at the plasma membrane of functional CFTR Cl^- channels (42, 43). Therefore, glycerol-treated CFPAC-1 cells were also used to test for the correlation between activation by cAMP of CFTR Cl^- channels and stimulation of Cl^-/HCO_3^- exchange. We used the patch clamp technique to measure whole cell Cl^- and K^+ currents and fluorometric measurements of pH_i using BCECF to determine HCO_3^- extrusion from the rate of recovery of pH_i after an alkaline load. Interactions of Cl^- and K^+ currents with Cl^-/HCO_3^- exchange activity were analyzed in both cell lines by studying the effects of *1)* raising intracellular cAMP or Ca^{2+} concentration, *2)* inhibition of Cl^- channels, *3)* cell membrane depolarization, *4)* ion substitution, and *5)* purinergic receptor stimulation with ATP.

MATERIALS AND METHODS

Materials

N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), nigericin, 4,4,-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether) N, N,N′,N′-tetraacetic acid (EGTA), gluconic acid, choline chloride, glycerol, forskolin, ionomycin, adenosine 3′:5′-cyclic monophosphate (cAMP), N^6,2′-O-dibutyryladenosine 3′:5′-cyclic monophosphate (DBcAMP), 3-isobutyl-1-methylxanthine (IBMX), glibenclamide, adenosine-5′-triphosphate magnesium salt, adenosine-5′-triphosphate sodium salt, tetramethylammonium hydroxide (TMA-OH) were from Sigma Chemical Company (St. Louis, Mo.); 2′7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethylester (BCECF-AM) was from Lambda GmbH (Graz, Austria). 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) was purchased from Calbiochem Chemical Company (La Jolla, Calif.). Culture media [Dulbecco's modified Eagle medium (DMEM), Iscove's modified Dulbecco's medium, and Leibowitz L-15 medium], fetal bovine serum (FBS), penicillin/streptomycin, and trypsin/EDTA were from Life Technologies, Inc. (Grand Island, N.Y.). A combination of 100 μmol/l DBcAMP, 100 μmol/l IBMX, and 3 μmol/l forskolin ('cAMPmix') was used to increase intracellular cAMP concentration (44).

Cell cultures

PANC-1 and CFPAC-1 cells were obtained from the American Type Culture Collection (Rockville, Md.). PANC-1 cells were grown in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). PANC-1 cells were used between passage 42 and 65. CFPAC-1 cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml). These cells were studied between passage 23 and 38. Cultures were incubated at 37°C in 5% CO_2 95% air atmosphere. The cells were suspended by washing with Ca^{2+}-Mg^{2+}-free PBS solution containing 2 mmol/l EDTA and plated on glass coverslip fragments. Cells were transferred to bicarbonate-free Leibowitz L-15 medium 1 h before use in patch clamp experiments.

Glycerol-pretreated CFPAC-1 cells were prepared by incubation for 24 h in Iscove's modified Dulbecco's medium supplemented with 10% glycerol (v/v), and glycerol was removed by stepwise diluting the glycerol-containing medium with fresh medium over the course of 90 min.

Intracellular pH (pH_i) measurement

Intracellular pH was measured as described (45) using the fluorescent intracellular sensor BCECF. In brief, BCECF was loaded into the cells in the form of its tetraacetoxymethyl ester derivative (BCECF-AM) (12 μM) by incubation for 15–20 min at 37°C. After washing for 10 min at 37°C in a BCECF-free medium, the cells were transferred into a thermostated (37°C) perfusion chamber placed on the stage of an Axiovert (Zeiss, Jena, Germany) inverted microscope. The microscope was equipped with a microfluorometer (Photon Technological Instruments, Monmouth Junction, N.J.), allowing for continuous dual wavelength excitation photometry. Intracellular pH was measured in single cells as the ratio of emission intensities at 530 nm after excitation at 495 nm (pH_i and concentration sensitive) and 440 nm (only concentration sensitive), respectively. Data were collected at 50 Hz chopping frequency and averaged every 2 s. After each experiment, internal dye calibration was performed by superfusing the cells with a medium containing high $[K^+]$ and the K^+/H^+ ionophore nigericin (12 μmol/l) at pH 6.8 and 7.6.

Determination of intrinsic intracellular buffer capacity

The intrinsic buffer capacity (β_i) is defined as the sum of intracellular buffers other than CO_2/HCO_3^-. Testing for buffer capacity by measuring changes of pH_i after the intracellular addition or removal of acid or base requires that pH_i is not affected by cell membrane acid or base transporters

(45). We performed measurements of β_i in HEPES-buffered, HCO_3^-- and Na^+-free solutions by exposing cells to NH_4Cl (30 mmol/l) to add base; after 3 min, we lowered external NH_4Cl concentration to 20 mmol/l to remove base. β_i was determined from calculated changes of $[NH_3+NH_4^+]_i$ and the associated changes of pH_i (45).

Measurement of Cl^-/HCO_3^- exchange activity

Cells were first superfused for 10 min with medium containing 25 mmol/l HCO_3^- and 5% CO_2, which results in intracellular equilibration of $[HCO_3^-]$ according to pH_i. Acute removal of CO_2/HCO_3^- from the superfusion medium by exposure to HEPES-buffered HCO_3^--free medium (see solutions below) results in depletion of intracellular CO_2 and sudden intracellular alkalinization. Intracellular pH recovers from this alkaline load toward the initial value. To prove that this pH_i recovery is due to the activity of Na^+-independent Cl^-/HCO_3^- exchange, we monitored pH_i after an alkaline load in the absence of external Cl^- (replacement with gluconic acid) and in cells preincubated with 0.5 mmol/l DIDS for 40 min. Possible dependence on Na^+ of the base extrusion mechanism was evaluated after preincubation with Na^+-free medium (replacement with choline). The rate of recovery from the intracellular alkaline load was determined by linear regression of the slope $\delta pH/\delta t$. For comparison between individual experiments, this slope was determined at the same range of pH_i (given in legends to Figs. 3 and 5). Experimental effects on Cl^-/HCO_3^- exchange activity were studied by preincubation of the cells for 10 min with either 'cAMPmix', NPPB, glibenclamide, high external K^+ concentration, ATP, or ionomycin.

Whole cell current recording

Whole cell currents were measured 24–48 h after plating the cells using patch clamp recording techniques (46). Studies were performed at room temperature (22°C) using NaCl-rich extracellular solution with 1 mmol/l free $[Ca^{2+}]$. Recording pipettes were pulled from VC-H075P glass (Terumo, Japan) on a micropipette puller P-87 (Sutter Instrument Co., Movato, Calif.) and had a resistance of 4–7 MΩ. The pipettes were filled either with KCl-rich solution or CsCl-rich solution with free $[Ca^{2+}]$ adjusted to ≈100 nmol/l (0.4 mmol/l $CaCl_2$ and 1 mmol/l EGTA). Data were recorded with an EPC-9 amplifier and digitized (3 kHz), stored on hard disk, and analyzed using Pulse+PulseFit version 7.4 programs (HEKA Elektronik GmbH, Lambrecht, Germany). Whole cell currents were measured at the holding potential (−40 mV) and during 100 ms square pulses of the test potential (−100 mV to +100 mV) in 20 mV increments, with 5 s intervals (polarity given for cell interior). For control bath and KCl-rich pipette solutions, the equilibrium potentials for K^+ (E_K) and Cl^- (E_{Cl}) were −86 mV and −1.3 mV, respectively. Therefore, membrane currents near these clamping voltages were considered to represent Cl^- and K^+ currents, respectively. Current-voltage relations were obtained after currents had stabilized 30 ms after applying voltage pulses. Whole cell currents were normalized for unit cell surface area by division by the whole cell membrane capacitance that ranged between 25 and 40 pF. Accordingly, current is presented as pA/pF. Corrections for pipette to bath liquid junction potentials were applied when asymmetrical solutions (KCl/NaCl or CsCl/NaCl) were used (4 mV and 5 mV, respectively).

Solutions

Buffers for pH experiments

Bicarbonate-free bath solution (HEPES) contained (mmol/l): NaCl 135, KCl 4.7, $MgSO_4$ 1, KH_2PO_4 1.2, $CaCl_2$ 1.5, HEPES 10, glucose 5, titrated to pH 7.4 with NaOH. Bicarbonate buffered solution (KRB) contained (mmol/l): NaCl 115, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1, $CaCl_2$ 1.5, $NaHCO_3$ 25, glucose 5, and was equilibrated with 5% CO_2.

Solutions for patch clamp experiments

Control NaCl-rich bath solution (mmol/l): NaCl 150, KCl 5, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10, glucose 5, titrated to pH 7.4 with NaOH, osmolality: 310–320 mosm/kg. Pipette solutions: KCl-rich solution contained (mmol/l): KCl 145, NaCl 5, MgCl 1, HEPES 10, $CaCl_2$ 0.4, EGTA 1, MgATP 2, titrated to pH 7.2 with KOH, osmolality: 280–290 mosm/kg. CsCl-rich pipette solution contained (mmol/l): CsCl 150, MgCl 1, HEPES 10, $CaCl_2$ 0.4, EGTA 1, MgATP 2, titrated to pH 7.2 with TMA-OH, osmolality: 280–290 mosm/kg. The osmotic difference between the pipette and bath solution was applied in order to prevent activation of volume-activated currents (47). With pipette solutions containing ATP, the efflux of ATP from the pipette could have stimulated purinergic membrane receptors when approaching the cell. This was avoided by first filling the pipette tip by dipping into an ATP-free pipette solution, followed by filling the pipette with the ATP-containing solution from the back. Stock solutions of NPPB, glibenclamide, forskolin and BCECF-AM were prepared in DMSO at 1000-fold the desired concentration. Nigericin was dissolved in ethanol.

Data analysis and statistics

We noticed some variation in electrical current and the rate of HCO_3^- extrusion between individual preparations that we could not attribute to the passage number or time after seeding. To avoid systematic errors in comparing one experimental condition with control or two different experimental conditions with each other, we performed the respective experiments on the same day, from one set of cell cultures and in random order. Data are acquired from single cells and given as mean values ± SE (n=number of cells). Differences between grouped experiments from one day are evaluated by the unpaired t test and are considered significant if $P<0.05$.

RESULTS

Intracellular pH and Cl^-/HCO_3^- exchange activity in PANC-1 and CFPAC-1 cells

Under control conditions, pH_i was higher in CFPAC-1 cells (7.34±0.02; n=16) than in control PANC-1 cells (7.16±0.05; n=10) ($P<0.005$). To test whether the high pH_i in CF cells may result from reduced HCO_3^- extrusion, we determined Cl^-/HCO_3^- exchange activity in both cell lines by measuring the rate of pH_i recovery after intracellular alkalinization. In both cell lines, pH_i recovered to baseline levels. Bicarbonate extrusion was Cl^- dependent, Na^+ independent, and inhibited by DIDS,

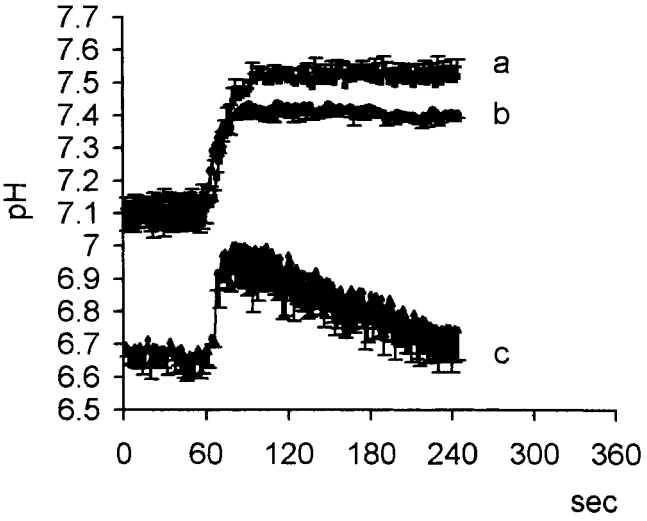

Figure 1. Measurement of pH_i in PANC-1 cells in order to test for the mechanism of base extrusion after an intracellular alkaline load. At 60 s, intracellular alkalinization was obtained by removal of HCO_3^- and CO_2 from the superfusion solution. a) No significant recovery of pH_i is obtained when extracellular Cl^- is also removed at the time of intracellular alkalinization. b) No significant recovery of pH_i is obtained when cells were preincubated for 40 min with 0.5 mmol/l DIDS. c) pH_i recovers at a normal rate toward baseline values in the absence of Na^+ (extracellular Na^+ being removed 40 min prior to alkalinization and absent throughout the experiment). Note low baseline pH_i in this condition. Data are presented as means ± SE, n=3 for a–c. Compare also Fig. 2.

consistent with Cl^-/HCO_3^- exchange (**Fig. 1**) as previously demonstrated in rat pancreatic duct cells (48, 49). The basal activity of this exchanger was higher in PANC-1 than in CFPAC-1 cells ($\delta pH/\delta t$=0.119±0.02/min; n=7 vs. $\delta pH/\delta t$=0.078±0.01/min; n=7; measured at pH=7.6; P<0.05). To compare rates of $\delta pH/\delta t$ in the two cell lines, we measured intracellular buffer capacity (β_i) using the NH_4Cl technique (45). Intracellular alkalinization was first obtained by exposing cells to 30 mmol/l NH_4Cl. Reducing external NH_4Cl from 30 to 20 mmol/l caused a sudden decrease of pH_i from 7.57 ± 0.10 to 7.45 ± 0.10 (n=5) and from 7.60 ± 0.06 to 7.47 ± 0.06 (n=4) corresponding to a β_i of 22.0 ± 3.6 mM/pH and 18.6 ± 1.8 mM/pH in PANC-1 and CFPAC-1 cells, respectively. The two values were not significantly different.

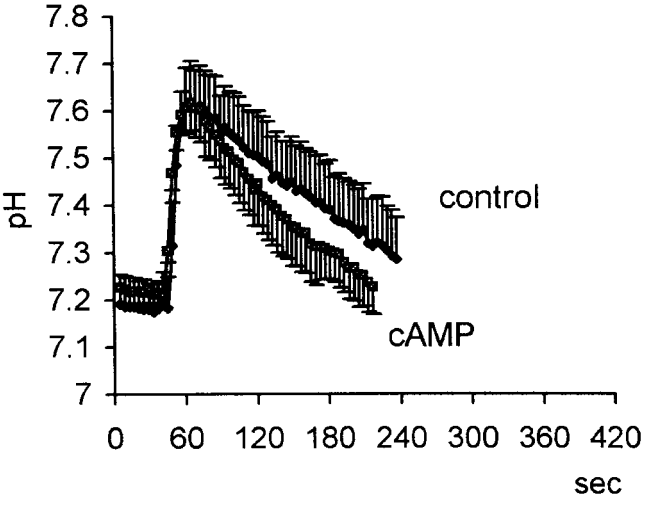

Figure 2. Measurement of pH_i in PANC-1 cells. Intracellular alkalinization was obtained by removal of HCO_3^- and CO_2 from the superfusion solution (at 50 s). The activity of Cl^-/HCO_3^- exchange was assessed from the rate of pH recovery under control condition (n=7) and after increasing intracellular cAMP concentration (n=6). Data are presented as means ± SE.

Increasing intracellular levels of cAMP and activating protein kinase A by administration of cAMPmix (44) led to a significant increase of Cl^-/HCO_3^- exchange activity in PANC-1 cells (**Fig. 2** and **Fig. 3**). In contrast, HCO_3^- extrusion can be stimulated in CFPAC-1 cells by an increase of $[Ca^{2+}]_i$ (**Fig. 4**, see below), whereas cAMPmix had no effect in these cells (**Fig. 5**). Because these two cell lines could differ in transport properties not related to differences in CFTR expression we also studied effects of cAMP in CFPAC-1 cells after induction by glycerol treatment

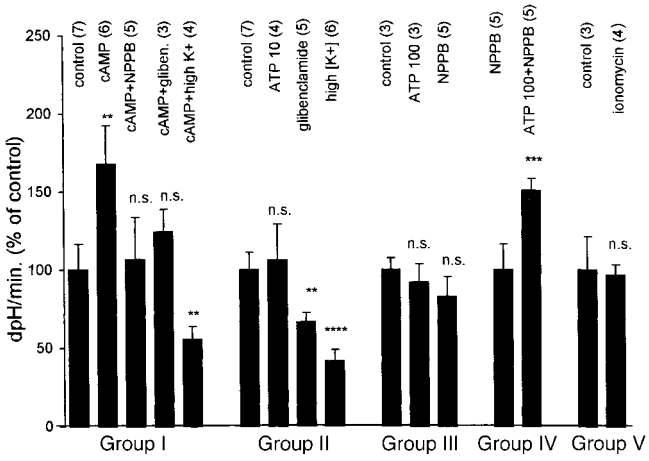

Figure 3. Cl^-/HCO_3^- exchange activity in PANC-1 cells is presented as the rate of recovery of pH_i after an alkaline load. Data from five groups of experiments are shown, each group with reference to the control rate (100%). Group I shows activation of Cl^-/HCO_3^- exchange by increasing intracellular cAMP and inhibition of the effect by the Cl^- channel blockers NPPB (10 μmol/l) and glibenclamide (100 μmol/l) and by exposure to high K^+ concentration (control value at pH_i=7.55: $\delta pH/\delta t$=0.119 ± 0.02/min). Group II shows lack of effect of 10 μmol/l ATP and inhibition of basal activity by glibenclamide (100 μmol/l) and by exposure to high $[K^+]_o$ (control value at pH=7.55: $\delta pH/\delta t$=0.173 ± 0.02/min). Group III: no significant effects of ATP (100 μmol/l) and NPPB (10 μmol/l) are seen for basal activity (control value at pH_i=7.25: $\delta pH/\delta t$=0.145 ± 0.011/min). Group IV: ATP (100 μmol/l) activates anion exchange in presence of NPPB (control value at pH_i=7.40: $\delta pH/\delta t$=0.085 ± 0.014/min). Group V: ionomycin did not increase the anion exchange activity (control value at pH_i=7.55: $\delta pH/\delta t$=0.173 ± 0.037/min). Data are presented as means ± SE. Number of cells is shown in parentheses for each group. *P<0.05; **P<0.025; ***P<0.01; ****P<0.005.

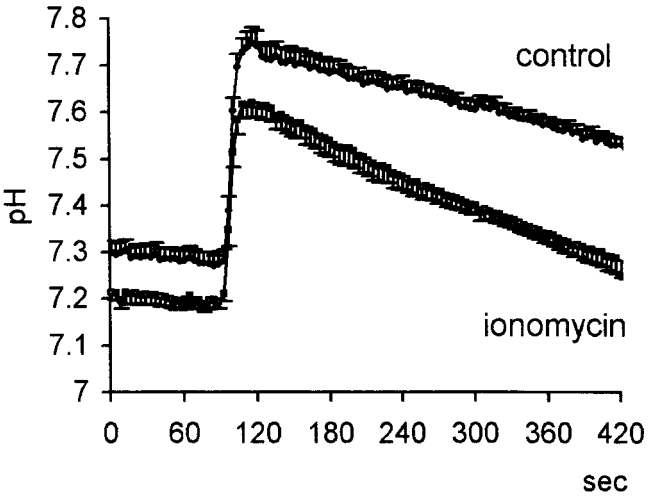

Figure 4. Measurement of pH_i in CFPAC-1 cells, experimental conditions as in Fig. 1. Note that basal pH_i is higher than in PANC-1 cells in control conditions (n=7) and that application of ionomycin (1 μmol/l) (n=6) reduces control pH_i and accelerates the rate of pH_i recovery after the alkaline load. Data are presented as means ± SE.

of plasma membrane expression of ΔF508-CFTR (42, 43). After glycerol pretreatment, CFPAC-1 cells assumed cAMP-induced HCO_3^- extrusion, δpH/δt increasing by ~ 2.5-fold by application of cAMPmix (Fig. 5). These data confirm that expression of CFTR in the plasma membrane is a prerequisite for stimulation of Cl^-/HCO_3^- exchange by cAMP.

To correlate Cl^-/HCO_3^- exchange activity in PANC-1 cells with simultaneous Cl^- flux via conductive pathways, we studied the effect of NPPB and glibenclamide, Cl^- channel blockers that inhibit CFTR at low concentration (28, 51, 52). Furthermore, we depolarized the membrane potential by exposing the cells to high extracellular K^+ concentration ($[K^+]_o$=70 mmol/l) with the aim to inhibit Cl^- efflux. As shown in Fig. 3, NPPB (10 μmol/l), glibenclamide (100 μmol/l), and application of high $[K^+]_o$ abolished the effect of cAMPmix on Cl^-/HCO_3^- exchange activity. In addition, 100 μmol/l glibenclamide and high $[K^+]_o$ reduced the basal bicarbonate output as well (Fig. 3). In accordance with measurements of membrane currents (see below), these data show that cAMP stimulates Cl^-/HCO_3^- exchange in PANC-1 cells by activation of NPPB- and glibenclamide-sensitive Cl^- channels. These channels allow for continuous recycling of Cl^- that is taken up by Cl^-/HCO_3^- exchange. Furthermore, the activation of these channels will render the $[Cl^-]_i$ dependent on the membrane potential; i.e.,: $[Cl^-]_i$ will be reduced on hyperpolarization, which in turn provides a steeper out-to-in Cl^- concentration gradient to activate Cl^-/HCO_3^- exchange.

To test whether Ca^{2+}-stimulated Cl^- efflux could provide another pathway for Cl^- recycling and for activation of the Cl^-/HCO_3^- exchanger, we studied the effects of purinergic receptor activation by application of ATP and of the calcium ionophore ionomycin (extracellular $[Ca^{2+}]$ being buffered to 1 μmol/l). In PANC-1 cells, application of ATP (10 and 100 μmol/l) had no significant effect on the high basal rate of anion exchange activity under control conditions, indicating a minor role of an additional ATP-activated Cl^- exit pathway in this cell line. Significant activation of base extrusion by ATP was seen only in the presence of NPPB, which is thought to inhibit basal activity of cAMP-dependent Cl^- channels, although inhibition of basal rate of base extrusion by NPPB remained below the level of significance (Fig. 3). In contrast, the basal Cl^-/HCO_3^- exchange activity in CFPAC-1 cells was highly activated by ATP at concentrations of 10 μmol/l and 100 μmol/l; increasing $[Ca^{2+}]_i$ by application of ionomycin (1 μmol/l) also doubled the rate of cell acidification (Figs. 4 and 5). This latter effect was inhibited by application of high $[K^+]_o$ but not by NPPB (10 μmol/l) (Fig. 5). These data indicate that both control and CF cells express Ca^{2+}-dependent Cl^- channels, but their activation stimulates the basal bicarbonate secretion through

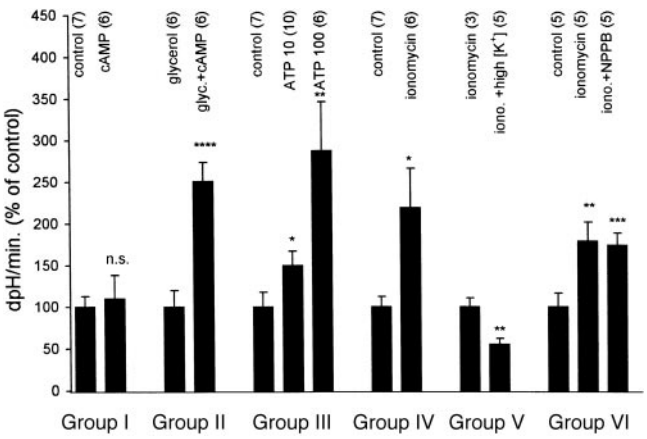

Figure 5. Cl^-/HCO_3^- exchange activity in CFPAC-1 cells is presented as the rate of recovery of pH_i after an alkaline load. Data from four groups of experiments are shown, each group with reference to the control rate (100%). Group I shows that administration of cAMP had no effect on the Cl^-/HCO_3^- exchange activity (control value at pH_i=7.55: δpH/δt=0.078±0.011/min). Group II: glycerol pretreatment restored cAMP effect on anion exchange (control value at pH_i=7.55: δpH/δt=0.035±0.007/min). Group III shows that both 10 μmol/l and 100 μmol/l ATP significantly stimulated the anion exchange activity (control value at pH_i=7.75: δpH/δt=0.048±0.009/min). Group IV: administration of 1 μmol/l ionomycin significantly increased the HCO_3^- secretion (control value at pH_i=7.6: δpH/δt=0.042±0.005/min). Group V: exposure to high extracellular $[K^+]$ inhibits the stimulatory effects of ionomycin (control value at pH_i=7.55: δpH/δt=0.123±0.013/min). Group VI: NPPB (10 μmol/l) had no effect on ionomycin stimulated HCO_3^- secretion. (control value at pH_i=7.5: δpH/δt=0.114±0.019/min). Data are presented as means ± SE Number of cells is shown in parentheses for each group. *P<0.05; **P<0.025; ***P<0.01; ****P<0.005.

TABLE 1. *Patch clamp whole cell recordings in PANC-1 cells*[a]

	Control (KCl) (10)	cAMP (KCl) (7)	High $[Ca^{2+}]_i$ (KCl) (4)
−85 mV	−2.49 ± 0.28	−6.3 ± 1.2***	−6.0 ± 1.42*
0 mV	+1.27 ± 0.22	+2.18 ± 0.73 ns	+3.33 ± 0.92 ns
+80 mV	+29.4 ± 4.4	+64.3 ± 13.91**	+28.4 ± 5.75 ns
Rev. pot.	−25.2 ± 3.9	−15.8 ± 1.9**	−29.7 ± 7.4 ns
	Glibenclamide (KCl) (7)	cAMP + glibenclamide (KCl) (4)	
−85 mV	−2.58 ± 0.61	−2.04 ± 0.46 ns	
0 mV	+1.88 ± 0.54	+1.12 ± 0.19 ns	
+80 mV	+42.6 ± 5.67	+52.6 ± 9.8 ns	
Rev. pot.	−30.1 ± 4.2	−29.0 ± 2.1 ns	
	Control (CsCl)[#] (6)	cAMP (CsCl) (5)	cAMP + NPPB (CsCl) (4)
−85 mV	−2.91 ± 0.48 ns	−4.37 ± 0.36**	−2.22 ± 0.8 ns
0 mV	+0.77 ± 0.22 ns	+1.09 ± 0.28 ns	+0.85 ± 0.23 ns
+80 mV	+4.13 ± 0.71****	+7.3 ± 0.46****	+3.7 ± 0.5 ns
Rev. pot.	−15.8 ± 4.4 ns	−17.1 ± 3.4 ns	−33.1 ± 9.5 ns

[a] Currents: pA/pF; positive values = outward current; reversal potential: mV; inside with respect to outside; KCl: KCl-rich pipette solution; CsCl: CsCl-rich pipette solution. Data represent the means ± SE. Number of cells is shown in parentheses. (ns = not significant; * $P < 0.05$; ** $P < 0.025$; *** $P < 0.005$; **** $P < 0.001$); [#] controls (CsCl) are compared to controls (KCl).

Cl^-/HCO_3^- exchange predominantly in CFTR-deficient CFPAC-1 cells.

Whole cell membrane Cl^- and K^+ currents in PANC-1 and CFPAC-1 cells

The experiments shown above indicate that activation of Cl^- currents by cAMP or Ca^{2+} stimulate Cl^-/HCO_3^- exchange in PANC-1 or CFPAC-1 cells, respectively, PANC-1 cells being responsive to cAMP with little effect of increasing $[Ca^{2+}]_i$, whereas CFPAC-1 cells respond to an increase of $[Ca^{2+}]_i$ only (except after pretreatment with glycerol). We used the patch clamp technique to correlate these effects of cAMP and $[Ca^{2+}]_i$ on HCO_3^- secretion with changes in whole cell membrane currents. Current/voltage (I/V) correlations were obtained using standard pipette (intracellular) solution with high KCl concentration, ~ 100 nmol/l free $[Ca^{2+}]$ and 2 mmol/l ATP, and a NaCl-rich bath (extracellular) solution. Application of these solutions determines the K^+ and Cl^- equilibrium potentials, E_K and E_{Cl} respectively, and clamping the membrane potential (V_m) to chosen values allows to obtain a measure of K^+ current (I_K) at V_m = 0 mV (near E_{Cl}, where the driving force for Cl^- current is absent; $V_m - E_{Cl}=0$) and of Cl^- current (I_{Cl}) at $V_m = -85$ (near E_K where K^+ current is absent; $V_m - E_K=0$). At the current reversal potential ($V_m=V_{rev}$) outward I_K ($I_K=g_K * (V_m - E_K)$) and inward I_{Cl} ($I_{Cl}=g_{Cl} * (V_m - E_{Cl})$) are of equal magnitude resulting in zero total membrane current and, depending on the relative values of membrane K^+ and Cl^- conductance, g_K and g_{Cl} respectively, V_{rev} will be close to E_K if $g_K \gg g_{Cl}$ or close to E_{Cl} if $g_{Cl} \gg g_K$. It may be noted that Cl^- inward current reflects conductive Cl^- efflux. In addition to I_{Cl} at $V_m = -85$ mV and to I_K at $V_m = 0$ mV, **Table 1** and **Table 2** also give the current reversal potentials as a measure of relative K^+ and Cl^- conductances together with the current at +80 mV, which in PANC-1 cells is predominated by an outwardly rectifying component of K^+ current (see below). In some experiments, intracellular KCl was completely replaced by CsCl. Thus, whole cell current is dominated by Cl^- over the entire voltage range.

Figure 6 shows the basal I/V relationship in PANC-1 cells and Table 1 gives the corresponding parameters for Cl^- and K^+ currents. A large outwardly rectifying current is observed at membrane voltages > +20 mV. This latter current component was suppressed by replacing K^+ with Cs^+ in the pipette solution which results in a near linear I/V relation, thus indicating the presence of outwardly rectifying K^+ channels in PANC-1 cells (Table 1).

In CFPAC-1 cells, the I/V relation was near linear over the entire voltage range studied (**Fig. 7**), current at −85 mV was small as compared to PANC-1 cells (−1.44±0.21 pA/pF; n=4 vs. −2.49±0.28 pA/pF; n=10, respectively) (P<0.01), and the reversal potential was more negative (−40.9±5.8 mV; n=4 vs. −25.2 ±. 3.9 mV; n=10, respectively) (P<0.05). Replacing K^+ with Cs^+ in the pipette solution resulted in a shift of the reversal potential toward E_{Cl} and reduced the current near E_{Cl}, demonstrating inhibition of K^+ current (Table 2). These data show that CFPAC-1 cells lack an outwardly rectifying component of K^+ current and that basal Cl^- current is smaller than in PANC-1 cells.

Figure 6 shows activation by intracellular cAMP of

TABLE 2. *Patch clamp whole cell recordings in CFPAC-1 cells*[a]

	Control (KCl) (4)	cAMP (KCl) (4)	High $[Ca^{2+}]_i$ (KCl) (5)
−85 mV	−1.44 ± 0.21	−1.55 ± 0.59 ns	−6.25 ± 0.6****
0 mV	+1.73 ± 0.5	+0.98 ± 0.24 ns	+3.74 ± 1.12 ns
+80 mV	+5.49 ± 0.66	+3.77 ± 1.35 ns	+15.2 ± 2.54***
Rev. pot.	−40.9 ± 5.8	−34.5 ± 6.1 ns	−28.2 ± 5.1 ns
	ATP (10 μM) (KCl) (4)	Glycerol (KCl) (4)	Glycerol + cAMP (KCl) (4)
−85 mV	−3.64 ± 0.24****	−1.10 ± 0.13	−3.2 ± 0.36****
0 mV	+3.42 ± 0.87 ns	+1.41 ± 0.22	+2.92 ± 0.96 ns
+80 mV	+13.3 ± 0.7***	+5.0 ± 0.33	+14.8 ± 2.94**
Rev. pot.	−35.4 ± 6.5 ns	−39.1 ± 6.9	−28 ± 8.1 ns
	Control (CsCl)[#] (4)	High $[Ca^{2+}]_i$ (CsCl) (4)	High $[Ca^{2+}]_i$ + NPPB (CsCl) (5)
−85 mV	−3.50 ± 0.98 ns	−8.90 ± 1.58**	−14.9 ± 0.9****
0 mV	+0.24 ± 0.06*	+0.83 ± 0.11****	+1.4 ± 0.18****
+80 mV	+4.48 ± 0.72 ns	+10.0 ± 1.2***	+16.0 ± 1.65***
Rev. pot.	−6.0 ± 1.3****	−7.9 ± 0.9 ns	−7.9 ± 1.5 ns

[a] Currents: pA/pF; positive values = outward current; reversal potential: mV; inside with respect to outside; KCl: KCl-rich pipette solution; CsCl: CsCl-rich pipette solution. Data represent the means ± SE. Number of cells is shown in parentheses. (ns = not significant; * $P < 0.05$; ** $P < 0.025$; *** $P < 0.005$; **** $P < 0.001$); [#] controls (CsCl) are compared to controls (KCl).

Cl^- and K^+ currents in PANC-1 cells: including 400 μmol/l cAMP into the pipette solution resulted in a significant increase of Cl^- current and of outwardly rectifying K^+ current (Table 1). Activation of Cl^- current by cAMP remained unaffected when the outward K^+ current was blocked by substituting intracellular K^+ by Cs^+ (Table 1), but additional application of NPPB (10 μmol/l) inhibited cAMP-activated Cl^- currents also (Table 1). Furthermore, application of glibenclamide (25 μmol/l) prevented the activation by cAMP of both Cl^- and K^+ currents (Table 1). In CFPAC-1 cells, intracellular application of cAMP had no effect on either Cl^- or K^+ currents (Table 2). However, after pretreatment of CFPAC-1 cells with glycerol, application of cAMP nearly tripled Cl^- current at −85 mV while K^+ current did not change significantly (Table 2).

In association with the experiments that showed stimulation of Cl^-/HCO_3^- exchange by an increase of $[Ca^{2+}]_i$ (see above), we tested for the presence of Ca^{2+}-activated membrane currents in PANC-1 and CFPAC-1 cells. We included Ca^{2+} in the pipette solution to raise $[Ca^{2+}]_i$ to a free concentration of ~1 μmol/l; in addition, we studied activation of membrane currents through stimulation of purinergic receptors by extracellular application of ATP.

Raising $[Ca^{2+}]_i$ resulted in an increase of Cl^-

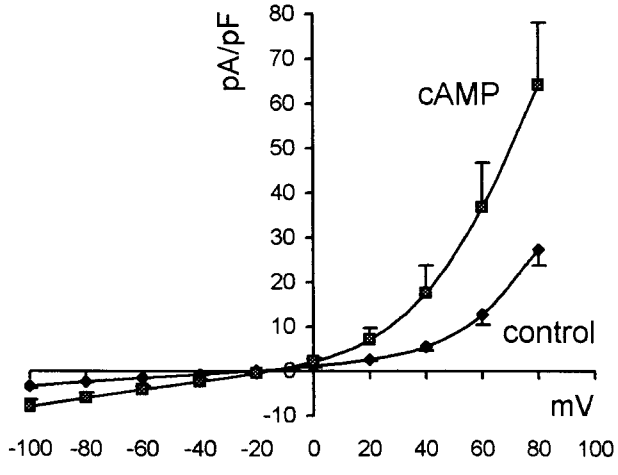

Figure 6. Whole cell patch clamp analysis of PANC-1 cells. Current-voltage relationships obtained under basal conditions (n=10) and after inclusion of 400 μmol/l cAMP in the patch pipettes (n=7). Data are presented as means ± SE.

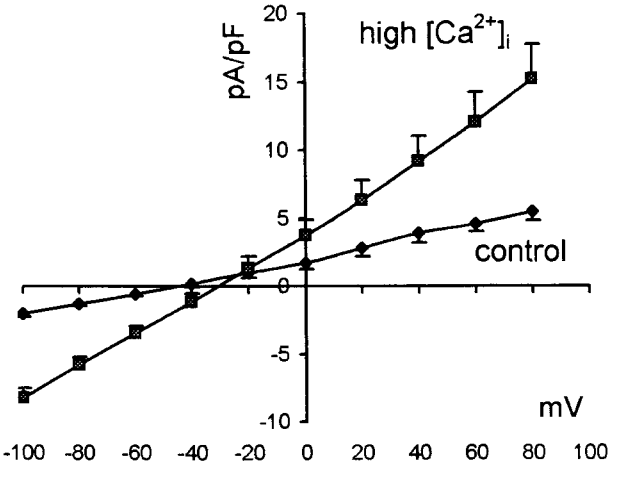

Figure 7. Whole cell patch clamp analysis of CFPAC-1 cells. Current-voltage relationships obtained under basal conditions (n=4) and after inclusion of 1 μmol/l Ca^{2+} in the patch pipettes (n=5). Note that the reversal potential is shifted toward E_{Cl}. Data are presented as means ± SE.

current in both cell lines (Tables 1 and 2). With respect to the basal level, the increase of Cl^- current by raising $[Ca^{2+}]_i$ was more pronounced in CFPAC-1 cells (Fig. 7). This increase of Cl^- current became particularly apparent when K^+ outward currents were suppressed by replacing intracellular K^+ by Cs^+ (Table 2). NPPB (10 μmol/l) was ineffective to inhibit this Ca^{2+}-activated Cl^- current. Similar to raising $[Ca^{2+}]_i$, extracellular application of 10 μmol/l ATP led to a substantial increase of the Cl^- current in CFPAC-1 cells (Table 2).

DISCUSSION

Pancreatic duct cells secrete a bicarbonate-rich fluid in response to secretin and other gastrointestinal hormones (25). The initial step for HCO_3^- secretion is the uptake of HCO_3^- ions into the epithelial cells, a process that can occur either by direct transport of HCO_3^- on Na^+:HCO_3^- cotransporters or by diffusion of CO_2 into the duct cells, followed by its hydration to H_2CO_3 and extrusion of protons via Na^+/H^+ exchangers or vacuolar H^+-ATPases (53). HCO_3^- ions are then secreted across the apical membrane by an electroneutral Cl^-/HCO_3^- exchanger. At a given pH_i, the rate at which this exchanger cycles will depend on the out-to-in Cl^- concentration gradient, where intracellular Cl^- concentration in turn depends on Cl^- efflux provided by opening of cAMP-activated apical Cl^- channels. Therefore, regulation of these channels appears to represent the main control point in the secretory mechanism.

In agreement with this concept, epithelial HCO_3^- secretion is reduced in patients with CF (26, 27). However, so far no study has directly addressed the relationships between CFTR deficiency and HCO_3^- secretion in the pancreatic duct epithelium. This study was therefore designed to directly compare Cl^-/HCO_3^- exchange activity and Cl^- conductance in two human pancreatic cell lines, one possessing functional CFTR (PANC-1) and the other being homozygous for ΔF508 (CFPAC-1). This mutation is characterized by impediment of surface expression of CFTR, a defect that can be overcome by chaperoning expression by glycerol treatment (42, 43).

These two cell lines appeared to represent useful models to study parallel activation of Cl^- channels and Cl^-/HCO_3^- exchange. The PANC-1 cell line retains a variety of normal differentiated epithelial cell characteristics, including expression of carbonic anhydrase (38) together with both cAMP- and Ca^{2+}-activated Cl^- conductance (54). The CFPAC-1 cell line is widely used as a model of CFTR-deficient cells (34, 35, 55–57). These cells have been shown to exhibit a high pH_i (56) but, as shown by short circuit current measurements, retain the ability of anion secretion on purinergic stimulation (33).

Both these cell lines exhibit Cl^-/HCO_3^- exchange activity that is dependent on extracellular Cl^-, independent of Na^+, and inhibited by DIDS. We show that elevation of intracellular cAMP increases HCO_3^- extrusion in PANC-1 cells, an effect that we could also observe after glycerol treatment of ΔF508-CFTR expressing cells. Purinergic stimulation was poorly effective in PANC-1 cells and detectable only in presence of NPPB. In contrast, HCO_3^- secretion was effectively stimulated in CFPAC-1 cells by both purinergic receptor occupancy and elevation of intracellular $[Ca^{2+}]$.

In interpreting these data we were faced with different concepts (proposed previously): *1)* the 'classical' view that electrogenic release of Cl^- through Cl^- channels and lowering of $[Cl^-]_i$ is a prerequisite of HCO_3^- secretion via Cl^-/HCO_3^- exchange (49). For this, cAMP-activated CFTR Cl^- channels could provide the necessary Cl^- conductance themselves or, alternatively, CFTR may activate other outwardly rectifying Cl^- channels (ORCC) that support Cl^-/HCO_3^- exchange (15), *2)* CFTR is directly responsible for HCO_3^- secretion without involving Cl^-/HCO_3^- exchange (58, 59), and *3)* regulation of Cl^-/HCO_3^- exchange by CFTR is independent of CFTR operating as a Cl^- channel (21).

To discriminate between these possibilities, we first showed that Cl^-/HCO_3^- exchange is the major if not the only mechanism of HCO_3^- extrusion in PANC-1 cells: this mechanism is dependent on extracellular Cl^-, independent of Na^+, and inhibited by DIDS. In addition, AE2 isoform is expressed in human fetal pancreatic ducts (60). We proceeded with studying *1)* the effects of inhibiting CFTR Cl^- conductance with NPPB or glibenclamide (48, 52) on HCO_3^- extrusion, *2)* effects of cell depolarization by high extracellular $[K^+]$ to identify electrogenic transport components, and *3)* effects on ion currents of elevating intracellular cAMP, intracellular $[Ca^{2+}]$, and of application of Cl^- channel blockers.

In all conditions tested, we find that activation or inhibition of HCO_3^- secretion is associated with a parallel increase or decrease of Cl^- conductance, respectively: raising intracellular cAMP lead to activation of Cl^- channels and HCO_3^- extrusion in PANC-1 cells and application of NPPB or glibenclamide inhibited both the cAMP-activated Cl^- conductance and cAMP-activated HCO_3^- extrusion. In addition, enabling ΔF508-CFTR expression in CFPAC-1 cells by glycerol treatment restored stimulatory effects of cAMP on both Cl^- conductance and HCO_3^- secretion. In CFPAC-1 cells, stimulation of HCO_3^- extrusion by raising $[Ca^{2+}]_i$ or by purinergic stimulation with ATP were both paralleled by an increase of Cl^- conductance. Neither Ca^+-activated

Cl^--conductance nor Ca^+-activated HCO_3^- extrusion was affected by NPPB. cAMP-activated HCO_3^- extrusion in PANC-1 cells and Ca^{2+}-activated HCO_3^- extrusion in CFPAC-1 cells were both abolished by cell depolarization. In addition, raising intracellular cAMP activated an outwardly rectifying current in PANC-1 cells. This current was suppressed by replacing K^+ with Cs^+ and inhibited by glibenclamide, indicating the presence of cAMP-activated K^+ channels but absence of ORCC. We therefore conclude that stimulation of Cl^-/HCO_3^- exchange depends on increase of Cl^- conductance, the latter being provided by CFTR in PANC-1 cells and by Ca^{2+}-activated Cl^- channels in CFPAC-1 cells, respectively. Thus, the lack of cAMP-mediated stimulation of HCO_3^- secretion in CFTR-deficient CFPAC-1 cells is compensated for by Ca^{2+}-activated Cl^- channels that are amenable to purinergic stimulation.

Our results also point to a specific role for K^+ channels in stimulation of HCO_3^- secretion: as noted above, PANC-1 cells exhibit a large outwardly rectifying K^+ current that is further activated by cAMP together with the increase of Cl^- conductance. These observations are in accordance with previous studies on isolated pancreatic ducts that showed that stimulation by secretin and VIP leads to sequential activation of K^+ and Cl^- currents, resulting in transient membrane hyperpolarization and followed by depolarization (24, 34). Note that expression of epithelial K^+ channels may be under the control of CFTR as shown in pancreas (13) and in kidney tubular cells (12). In CFTR-deficient CFPAC-1 cells, cAMP failed to activate both Cl^- and K^+ currents. However, glycerol treatment of these cells restored cAMP-dependent Cl^- conductance without effect on K^+ current. Notwithstanding, Lousourn at al. have shown that transfection of CFPAC-1 cells with wild-type CFTR induced a cAMP-dependent K^+ current (13), possibly, suggesting that the ΔF508 mutation is less efficient in supporting K^+ channel expression. On the other hand, CFPAC-1 cells exhibited a large K^+ current in the presence of extracellular ATP or at high $[Ca^{2+}]_i$ that was significantly suppressed by replacing intracellular K^+ with Cs^+ (Table 2). This current appeared sufficient to provide for charge neutralization for Cl^- efflux through Ca^{2+}-activated channels.

In **Fig. 8** we have summarized the proposed roles of Cl^- and K^+ channels in supporting bicarbonate secretion via Cl^-/HCO_3^- exchange. The apical membrane is shown on the top, including electroneutral Cl^-/HCO_3^- exchange together with cAMP-activated (CFTR; left) and Ca^{2+}-activated Cl^- channels, as found in PANC-1 and CFPAC-1 cells, respectively. Activation of these channels serves for recycling of Cl^- that enters the cell through Cl^-/HCO_3^- exchange, thus permitting for HCO_3^- secretion at a high rate. Channels and transporters of the basolateral membrane are shown in the lower part. K^+ channels play a significant role in the activation of Cl^-/HCO_3^- exchange: activation of a K^+ efflux will tend to hyperpolarize the membrane potential, which, as a consequence, stimulates Cl^- efflux through apical Cl^- channels, thus reducing $[Cl^-]_i$ and providing a steeper Cl^- concentration gradient as the driving force for electroneutral Cl^-/HCO_3^- exchange. Viewed differently, K^+ outward current will provide charge neutralization for efflux of Cl^- through apical Cl^- channels, which is required for recycling of Cl^- that enters the cell through Cl^-/HCO_3^- exchange (61). Furthermore, in this interplay between channels and carriers, basolateral Na^+/K^+-ATPase will serve as the primary pump to refuel intracellular K^+. Simultaneously, the pump will maintain intracellular Na^+ concentration at a low level and thus provide the driving force for stimulation of either Na^+/H^+ exchange or Na^+:HCO_3^- symport mechanisms that lead to accumulation or generation of intracellular HCO_3-. The latter transporter exhibits reduced activity in CF (20). In this integrated view, stimulation of HCO_3^- secretion will result in simultaneous conductive apical Cl^- efflux (inward current) and basolateral K^+ efflux. Combined, these ion fluxes result in an apical-to-basolateral transcellular current

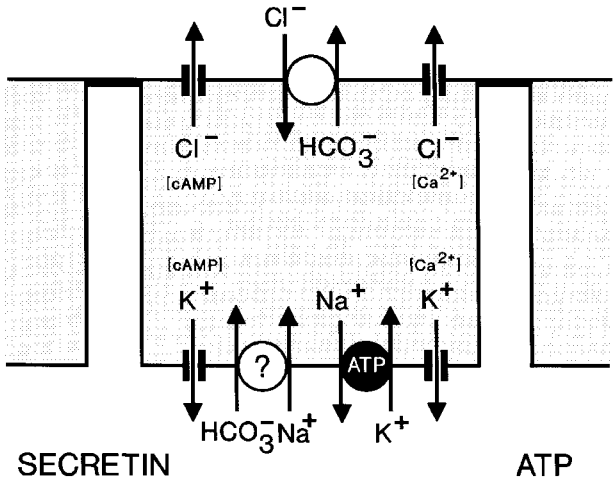

Figure 8. Model of pancreatic duct cell to show apical membrane bicarbonate secretion (upper part) by interaction of Cl^- and K^+ channels and apical Cl^-/HCO_3^- exchange. The basal membrane (lower part) contains the Na^+/K^+-pump that establishes an out-to-in Na^+ concentration gradient that serves as a driving force for intracellular accumulation of HCO_3^- via Na^+:HCO_3^- symport or Na^+/H^+ exchange. Stimulation of bicarbonate secretion by secretin (left) and ATP (right) is shown. Secretin activates K^+ and Cl^- channels via generation of intracellular cAMP. Activation of basolateral K^+ channels hyperpolarizes the cell favoring apical Cl^- efflux through cAMP-activated Cl^- channels (CFTR). These channels allow for recycling of Cl^- that is taken up into the cell by Cl^-/HCO_3^- exchange. ATP acts via purinergic receptors (not shown) resulting in activation of Ca^{2+}-dependent K^+ and Cl^- channels. Activation of Ca^{2+}-dependent Cl^- channels can substitute in sustaining Cl^-/HCO_3^- exchange if CFTR is inoperative.

that is the basis for assessing secretion by short circuit current measurements.

In Fig. 8, secretin is depicted as the classical agonist for cAMP-mediated stimulation of HCO_3^- secretion. This mechanism is the prevailing one if CFTR is present in the apical cell membrane (data presented for PANC-1 cells). For CFTR-deficient pancreatic duct cells (CFPAC-1), our results show that Ca^{2+}-dependent apical Cl^- channels provide an alternative mechanism of stimulating apical Cl^-/HCO_3^- exchange. We also show that this mechanism is activated by purinergic stimulation. This alternative activation of cAMP and Ca^{2+}-dependent Cl^- channels appears analogous to the recent observation that the decrease of cAMP-dependent Cl^- flux in gallbladder epithelium of CF patients is compensated by reciprocal increase of ATP-dependent flux (62).

This Ca^{2+}-dependent mechanism of Cl^- channel activation is of considerable interest. CFTR appears to modulate ATP secretion from the cells (15, 17) whereby extracellular ATP could serve as an autocrine purinergic agonist to stimulate Ca^{2+}-dependent transport. Furthermore, purinergic receptor agonists have been shown to enhance Cl^- transport by increasing $[Ca^{2+}]_i$ in a number of epithelia such as airway epithelial cells (63), bile duct cells (64), gallbladder (62), and pancreatic duct cells deficient in CFTR (65). In the biliary system, apical purinergic receptors have been identified and ATP is present in bile in micromolar concentrations, perhaps explaining why cholestatic symptoms of CF are delayed in their appearance (66). Trials using UTP and ATP for treatment of pulmonary complications of CF have been promising (67). The demonstration of Ca^{2+}-activated Cl^- channels in freshly isolated human pancreatic duct cells (68) and of P_{2Y2} receptors in both CFPAC-1 cells (57) and dog pancreatic duct cells (31) suggests that similar strategies can be considered for treatment of pancreatic disease in CF.

In conclusion, our data confirm the presence of cAMP- and Ca^{2+}-activated Cl^- transport in PANC-1 and CFPAC-1 cells, respectively, and we show that stimulation of HCO_3^- secretion via Cl^-/HCO_3^- exchange is linked to activation of the respective Cl^- channels. Ca^{2+}-dependent Cl^- channels and HCO_3^- secretion in CFTR deficient cells can be activated by purinergic stimulation, a mechanism that could be exploited by devising pharmacological strategies to bypass the secretory defect in cystic fibrosis and related pancreatic diseases (6). FJ

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