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Development of Substituted Benzo[c]quinolizinium Compounds as Novel Activators of the Cystic Fibrosis Chloride Channel*

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Chloride channels play an important role in the physiology and pathophysiology of epithelia, but their pharmacology is still poorly developed. We have chemically synthesized a series of substituted benzo[c]quinolizinium (MPB) compounds. Among them, 6-hydroxy-7-chlorobenzo[c]quinolizinium (MPB-27) and 6-hydroxy-10-chlorobenzo[c]quinolizinium (MPB-07), which we show to be potent and selective activators of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. We examined the effect of MPB compounds on the activity of CFTR channels in a variety of established epithelial and nonepithelial cell systems. Using the iodide efflux technique, we show that MPB compounds activate CFTR chloride channels in Chinese hamster ovary (CHO) cells stably expressing CFTR but not in CHO cells lacking CFTR. Single and whole cell patch clamp recordings from CHO cells confirm that CFTR is the only channel activated by the drugs. Ussing chamber experiments reveal that the apical addition of MPB to human nasal epithelial cells produces a large increase of the short circuit current. This current can be totally inhibited by glibenclamide. Whole cell experiments performed on native respiratory cells isolated from wild type and CF null mice also show that MPB compounds specifically activate CFTR channels. The activation of CFTR by MPB compounds was glibenclamide-sensitive and 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid-insensitive. In the human tracheal gland cell line MM39, MPB drugs activate CFTR channels and stimulate the secretion of the antibacterial secretory leukoproteinase inhibitor. In submandibular acinar cells, MPB compounds slightly stimulate CFTR-mediated submandibular mucin secretion without changing intracellular cAMP and ATP levels. Similarly, in CHO cells MPB compounds have no effect on the intracellular levels of cAMP and ATP or on the activity of various protein phosphatases (PP1, PP2A, PP2C, or alkaline phosphatase). Our results provide evidence that substituted benzo[c]quinolizinium compounds are a novel family of activators of CFTR and of CFTR-mediated protein secretion and therefore represent a new tool to study CFTR-mediated chloride and secretory functions in epithelial tissues.

Cystic fibrosis (CF),[1] the most common fatal genetic disease is characterized by defective chloride transport across epithelia of the airways, exocrine ducts, and intestine as well as viscous epithelial mucous secretions (1–3). The mutated gene that causes CF encodes the cystic fibrosis transmembrane conductance regulator (CFTR) (3). CFTR, which belongs to the ABC (ATP binding cassette) family of transporters (3), is a regulated chloride channel that plays a key role in the hormone-dependent ion transport across epithelia in a variety of different species and organs (reviewed in Ref. 4). CFTR also regulates secretion of mucins and serous proteins in epithelial cells (5, 6). Under normal physiological conditions, opening of the CFTR channel is triggered by secretagogues that elevate intracellular cAMP (4, 7), resulting in protein kinase A-mediated phosphorylation at multiple sites on the R domain (8). In CF, mutations in the gene produce proteins that are not correctly processed

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese hamster ovary; MPB, benzo[c]quinolizinium compounds; MPB-02, 6-amino-10-chlorobenzo[c]quinolizinium chloride; MPB-04, 6-amino-7-chlorobenzo[c]quinolizinium chloride; MPB-07, 6-hydroxy-10-chlorobenzo[c]quinolizinium; MPB-27, 6-hydroxy-7-chlorobenzo[c]quinolizinium; I_{sc}, short circuit current; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; SLPI, secretory leukoproteinase inhibitor; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; WT, wild type; pF, picofarads; cpt-cAMP, 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate.

and fail to traffic to the plasma membrane, have a reduced conductance, or are incorrectly regulated by physiological stimuli (4, 9–11). This disrupts the normal transport of salt, water, and proteins across epithelial tissues, which leads to the production of thickened secretory product and to progressive obstruction of secretory ducts leading to organ dysfunction (1, 2).

Great effort has been made during the past 5 years to identify suitable CFTR chemical activators. Such substances would benefit patients by increasing the fluidity of secretions. These chemicals include those which can affect CFTR indirectly by interacting with parts of the cAMP signaling mechanism such as phosphodiesterase inhibitors (5, 11) and phosphatase inhibitors (12–15). Direct activation of CFTR has been postulated when using the tyrosine kinase inhibitor flavonoid drug genistein (16, 17), xanthine derivatives (15, 18) including the adenosine receptor antagonist CPX (19), and the K^+ channel activators benzimidazolone compounds NS004 (20, 21) and 1-EBIO (21). However, the mode of action and the specificity of these latter activators is still debated.

The goal of our study was to design new activators of CFTR channels. We have chemically synthesized molecules and tested them using an iodide efflux assay adapted for the study of CFTR channels in stably transfected Chinese hamster ovary (CHO) cells. Selected compounds were then evaluated for actions on chloride transport, secretion of serous proteins, and mucins within a consortium of seven laboratories, and results were collected and compared. Here we report on the development of chemicals belonging to the benzo[c]quinolizinium family (named MPB) (22), which we show are selective activators of CFTR channels. To our knowledge, this strategy is the first to be reported in the field of the pharmacology of chloride channels.

EXPERIMENTAL PROCEDURES

Chemical Synthesis (Fig. 1A)

6-Amino-10-chlorobenzo[c]quinolizinium chloride (MPB-02; Fig. 1B)—After stirring during 30 min at 0 °C, a mixture of 2.23 g (0.022 mol) of diisopropylamine in tetrahydrofurane (30 ml) and 13.75 ml of a 1.6 M solution of BuLi was cooled to −40 °C before the addition of 1.86 g (0.02 mol) of 2-methylpyridine. After 30 min, 3.44 g (0.02 mol) of 2,3-dichlorobenzonitrile in 20 ml of tetrahydrofurane was added. After stirring for 1 h at −40 °C, the solution was further stirred for 20 h at 20 °C and hydrolyzed with 10 ml of water. The organic layer was dried over Na_2SO_4, concentrated under vacuum, and warmed to 200 °C under N_2 for 15 min. The residue was washed with propanone and purified by flash chromatography on Al_2O_3 using ethyl acetate-ethanol (70/30) as eluent to give 1.10 g (20%) of a yellow powder: melting point >260 °C (decomposition). Anal. C_{13} H_{10} Cl_2 N_2, 0.5 H_2O: C, 56.96; H, 4.04; N, 10.22; Found: C, 56.83; H, 4.19; N, 10.21. IR (KBr): 3430, 3281, 3115, 1635. ^{1}H NMR (Me_2SO-d_6): σ 9.2 (d, J = 7 Hz, 1H, H1), 9.0–7.5 (m, 6H), 7.4–6.5 (m, 1H + NH_2).

6-Amino-7-chlorobenzo[c]quinolizinium chloride (MPB-04, Fig. 1B)—MPB-04 was synthesized using the procedure described for the formation of MPB-02 but starting from 2,6-dichlorobenzonitrile. Yellow powder, melting point >260 °C, yield: 42%. Anal. C_{13} H_{10} Cl_2 N_2, 2H_2O: C, 51.84; H, 4.68; N, 9.30. Found: C, 51.75; H, 4.27; N, 8.95. IR (KBr): 3417, 3176, 1643, 1596, 1451. ^{1}H NMR (Me_2SO-d_6): σ 10.1 (d, J = 7 Hz, 1H, H1), 9.4 (m, 1H), 8.6–8.2 (m, 5H), 8.1–7.5 (m, 1H + NH_2). Mass spectrum (EI, *m/z*): 228 (50), 201 (100), 192 (12), 166 (17), 139 (8).

6-Hydroxy-10-chlorobenzo[c]quinolizinium chloride (MBP-07, Fig. 1B)—MPB-07 was synthesized using the procedure described for the formation of MPB-02. After the addition of 2,3-dichlorobenzonitrile, the solution was stirred for 1 h at −40 °C and 20 h at 20 °C and hydrolyzed with 20 ml of H_2O, and the pH was adjusted to 2 with H_2SO_4. The solvent tetrahydrofurane was evaporated, and the solution was warmed to reflux with stirring during 3 h. The solution was then extracted with $CHCl_3$ (3 × 30 ml). The organic layer was dried over Na_2SO_4, concentrated under vacuum, and purified by column chromatography with toluene as eluent. Pure product was then warmed under N_2 to 200 °C for 15 min. The residue was washed with CH_2Cl_2 and recrystallized in ethanol. Cream powder; melting point = 210–220 °C (decomposition), yield: 42%. Anal. C_{13} H_9 Cl_2 N O, 0.5 H_2O: C, 56.75; H, 3.66; N, 5.09; Found: C, 56.25; H, 3.31; N, 4.78. IR (KBr): 3143, 3029, 2416, 1634, 1590, 1487. ^{1}H NMR (Me_2SO-d_6): σ 9.6 (d, J = 7 Hz, 1H, H1), 8.4–7.3 (m, 7H + OH). Mass spectrum (EI, *m/z*): 229 (91) (M-HCl), 201 (100), 166 (82), 139 (67).

6-Hydroxy-7-chlorobenzo[c]quinolizinium chloride (MPB-27, Fig. 1B)—MPB-27 was synthesized using the procedure described for the formation of MPB-07 but starting from 2,6-dichlorobenzonitrile. Cream powder, melting point = 240–250 °C (decomposition), yield: 31%. Anal. C_{13} H_9 Cl_2 N O: C, 58.67; H, 3.41; N, 5.26. Found: C, 58.68; H, 3.51; N, 5.24. IR (KBr): 3097, 3045, 2396, 1641, 1608, 1593, 1455. ^{1}H NMR (Me_2SO-d_6): σ 9.7 (d, J = 7 Hz, 1H, H1), 9.0–8.7 (m, 1H), 8.1–7.8 (m, 4H), 7.8–7.4 (m, 2H + OH). Mass spectrum (EI, *m/z*): 229 (M-HCl) (59), 201 (100), 166 (21), 139 (14).

Iodide Efflux Experiments

Chinese hamster ovary (CHO-K1) cells stably transfected with either pNUT vector alone (CFTR(−) CHO cells) or pNUT containing wild type CFTR (CFTR(+) CHO cells) were provided by J. R. Riordan and X.-B. Chang (Mayo Clinic, Scottsdale, AZ) (15, 23, 24). Cells cultured at 37 °C in 5% CO_2 were maintained in α-minimal essential medium containing 7% fetal bovine serum, antibiotics (50 IU of penicillin/ml and 50 μg/ml streptomycin), and 100 μM methotrexate (all from Sigma).

CFTR chloride channel activity was assayed by measuring iodide (^{125}I) efflux from transfected CHO cells as described previously (12, 18). All experiments were performed at 37 °C. Cells grown for 4 days in 12-well plates were washed twice with 2 ml of modified Earle's salt solution (solution B) containing 137 mM NaCl, 5.36 mM KCl, 0.4 mM Na_2HPO_4, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.5 mM glucose, and 10 mM HEPES, pH 7.4. Cells were then incubated in B medium containing 1 μM KI (1 μCi of $Na^{125}I$/ml, NEN Life Science Products) for 30 min at 37 °C. After washing, cells were incubated with 1 ml of solution B. After 1 min, the medium was removed to be counted and was quickly replaced by 1 ml of the same medium. This procedure was repeated every 1 min for 11 min. The first two aliquots were used to establish a stable base line in efflux buffer alone. B medium containing the appropriate drug was used for the remaining aliquots. At the end of the incubation, the medium was recovered, and cells were solubilized in 1 N NaOH. The radioactivity was determined using a γ-counter (LKB). The total amount of ^{125}I (in cpm) at time 0 was calculated as the sum of cpm counted in each 1-min sample plus the cpm in the NaOH fraction. The fraction of initial intracellular ^{125}I lost during each time point was determined, and time-dependent rates of ^{125}I efflux were calculated according to Venglarik *et al.* (25) from $\ln(^{125}I_{t_1}/^{125}I_{t_2})/(t_1 - t_2)$, where $^{125}I_t$ is the intracellular ^{125}I at time t, and t_1 and t_2 are successive time points (25). Curves were constructed by plotting rate of ^{125}I efflux *versus* time. Data are presented as the mean ± S.E. of n separate experiments. Differences were considered statistically significant using the Student's t test when the p value was <0.05.

Patch Clamp Recordings from CHO and MM39 Cells

CHO or MM39 cells were plated on 35-mm Petri dishes and cultured at 37 °C in 5% CO_2 for 1–4 days before use. Single channel currents were recorded from cell-attached patches with a List EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). Experiments were performed at room temperature. Results were displayed conventionally with inward currents (outward flow of anions) indicated by downward deflections. Potentials were expressed as the bath potential minus the patch electrode potential. The pipette solution contained 150 mM choline-Cl, 2 mM $MgCl_2$, and 10 mM TES (pH 7.4); the bath contained 145 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, and 10 mM TES (pH 7.4). Other details appeared elsewhere (18). Data are presented as the mean ± S.E. of n separate experiments.

Whole cell currents were recorded with an RK300 patch-clamp amplifier (Biologic, France). The current-voltage relationships were determined from step voltage protocols. The membrane potential was first held at −40 mV and then voltage-clamped over the range ±80 mV in steps of 20 mV. Currents were low pass-filtered at 3.3 kHz, digitized on-line at 4 kHz, and stored on the computer hard disk. They were analyzed off-line with the pCLAMP 5.5.1 software package (pCLAMP, Axon Instruments). Pipettes with resistance of 2–5 megaohms were pulled from borosilicate glass capillary tubing (GC150-TF10, Clark Electromedical Inc., Reading, UK) using a two-step vertical puller (Narishige, Japan). They were connected to the head stage of the amplifier through an Ag/AgCl pellet. Seal resistance ranging from 3 to 30 gigaohms were obtained. The pipette solution contained 145 mM CsCl, 5 mM NaCl, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM HEPES (pH 7.2). The external solution consists of 150 mM NaCl, 2 mM $CaCl_2$, 2 mM

$MgCl_2$, 10 mM HEPES (pH 7.4). All experiments were performed at room temperature.

Cells were stimulated with forskolin or an appropriate compound (dissolved in Me_2SO; final Me_2SO concentration 0.1%) at the concentration indicated under "Results." In control experiments, the currents were not altered by Me_2SO.

Whole Cell Recordings from Isolated Murine Ciliated Nasal Cells

Mice of either sex from a Balb/c breeding colony at the University of Newcastle upon Tyne or transgenic CF null mice (26) were used for these experiments (three wild type and two CF null animals). Ciliated respiratory cells were obtained using an isolation technique that has been fully described previously (27). In brief, nasal epithelium was incubated with 0.05% protease XIV (Sigma) for 24–30 h at 4 °C, and single ciliated respiratory cells were teased from the epithelium (27). Our criteria for cell viability were (i) a clear, bright, phase-contrast image and (ii) beating cilia (27).

Patch clamp recordings were made at room temperature either from single cells or small groups of cells (≤7). Whole cell currents were recorded with an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany). To obtain *I*/V relationships, the membrane potential was held at 0 mV and then voltage-clamped over the range ±80 mV in steps of 20 mV with each voltage step lasting 500 ms. Data were filtered at 1 kHz and sampled at 2 kHz with a Cambridge Electronic Design 1401 interface (CED, Cambridge, UK) and stored on the computer hard disk. The input capacitance of the cells was measured using the analogue circuitry of the amplifier and used to calculate current density which is expressed as pA/pF. Junction potentials were measured, and the appropriate corrections were applied to V_m.

The pipette solution contained 120 mM *N*-methyl-D-glutamine-Cl, 2 mM $MgCl_2$, 2 mM EGTA, 1 mM ATP, 10 mM HEPES, pH 7.2 (calculated free Ca^{2+} concentration <1 nM). The standard bath solution contained 149.5 mM *N*-methyl-D-glutamine Cl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4. As we have previously found for murine pancreatic duct cells (28), in order to detect CFTR currents, airway cells had to be pretreated with forskolin (1 μM), dibutyryl cAMP (100 μM), and 3-isobutyl-1-methylxanthine (100 μM) before whole cell recording was established. Preliminary experiments showed that CFTR currents were only detected if the cAMP stimulants were included in the protease solution used to isolate the respiratory cells (24–30 h at 4 °C). An identical protocol was employed for the MPB compounds. Cells remained viable after exposure to the MPB compound as judged by the criteria listed above.

Significance of difference between means was determined using analysis of variance followed by Dunn's multiple comparison test. The significance of difference between the number of cells responding to a particular maneuver was assessed using the χ^2 test. The level of significance was set at $p \leq 0.05$. All values are expressed as mean ± S.E. (number of observations).

Short Circuit (I_{sc}) Measurements of Human Nasal Epithelial Cells

The method for the primary culture of nasal epithelial cells has been described elsewhere (29, 30). Briefly, nasal polyps were digested overnight in a solution containing protease XIV. Detached epithelial cells were seeded at high density (3×10^6 cells/cm^2) on Snapwell (Costar) permeable supports. Culture medium was Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) plus 5% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin for the first 24 h. Subsequently, this medium was replaced with one containing 2% Ultroser G (Life Technologies, Inc.) instead of fetal calf serum.

Ussing chamber experiments were performed 4–5 days after cell seeding. At this time, cell monolayers displayed a transepithelial potential difference of −52.4 ± 1.7 mV and an electrical resistance of 1007 ± 36 ohms · cm^2. The Snapwell cups were mounted in a modified Ussing chamber (Costar) filled on both sides with 5 ml of a Krebs bicarbonate solution containing 126 mM NaCl, 0.38 mM KH_2PO_4, 2.13 mM K_2HPO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 24 mM $NaHCO_3$, 10 mM glucose, and 0.04 mM phenol red. During the experiments, this solution was continuously bubbled with 5% CO_2, 95% air and kept at 37 °C. The epithelium was short circuited with a voltage clamp (558-C5, Bioengineering, The University of Iowa) connected to apical and basolateral chambers with Ag/AgCl electrodes. The potential difference and the fluid resistance between potential sensing electrodes was compensated. The short circuit current (I_{sc}) was recorded simultaneously on a chart recorder (L6512, Linseis) and a computer Power Macintosh equipped with a MacLab/200 converter.

Measurement of Mucin Secretion, Cellular cAMP, and ATP in Rat Submandibular Acini

Methods for isolating preparations of rat submandibular acini and for measurement of mucin secretion have been described elsewhere (6, 31). Briefly, acini were pulse-chase-labeled with [^{3}H]glucosamine (5 μCi/ml), suspended in KHB containing 20 mg/ml bovine serum albumin, and incubated under experimental conditions at 37 °C. [^{3}H]glucosamine-labeled mucins, released into the medium at zero time and after 60 min, were acid-precipitated using a combination of 10% trichloroacetic acid and 0.5% phosphotungstic acid. The precipitates were washed, and their radioactivity was measured as described. The majority of the radioactivity in the trichloroacetic acid/phosphotungstic acid precipitate has characteristics of mucin in both basal and stimulated rat submandibular acinar cell secretions (31). The protein content of cell pellets was determined using the Bio-Rad protein assay kit, and mucin release is expressed as a percentage of basal secretion to take account of variation in unstimulated mucin release between experiments.

For cAMP and ATP measurement, acini were incubated for 5 and 60 min, respectively, at 37 °C in the presence or absence of test compounds. Aliquots of acini suspensions (0.25 ml) were added to an equal volume of ice-cold trichloroacetic acid (20%), extracted and assayed using a specific radioimmunoassay kit for cAMP (Amersham Pharmacia Biotech) and a luminometric assay using firefly luciferin-luciferase for ATP, as described previously (6).

Assay for SLPI Secretion in MM39 Cells

Confluent cultures of the human tracheal gland MM39 cell line (32, 33) grown on 24-well plates were rinsed four times for 1 h with serum-free culture medium and then exposed for 30 min to nucleosides or agents. 40 μl of the culture medium was harvested, and the secretion of the secretory leukoproteinase inhibitor SLPI was directly measured by enzyme-linked immunosorbent assay (34). The polyclonal antibodies used were highly specific and able to recognize the molecule even complexed to mucins or to proteases, allowing accurate detection of SLPI in the culture medium. The SLPI secretory rate determined from quadruplicate assays, was expressed as the ratio of SLPI secreted in the presence of agonists to that secreted in control wells to which only vehicle solutions were added. Vehicle additions were shown to be ineffective on SLPI secretion by MM39 cells.

Measurement of CHO Cellular cAMP and ATP

CHO cells grown for 4 days in 12-well plates were incubated in the presence or absence of test compounds. After a 5-min incubation period at 37 °C, the reaction was stopped by adding 55 μl of 11 N perchloric acid. A radioimmunoassay kit (RIANEN kit, NEN Life Science Products) was used to determine cAMP levels. ATP was measured (in triplicate) using the luciferin-luciferase method by a bioluminescent kit (CLS Test Combination from Roche Molecular Biochemicals). In order to compare the effect of different drugs, test data are expressed as percentage of ATP content of cells incubated in the absence of drugs.

Assay for Protein Phosphatase Activities

PP1, PP2A, and PP2C were assayed from a transfected CHO extract obtained after centrifugation of cell homogenate at 20,000 × *g*. PP1 and PP2A activities were determined by measuring the release of [^{32}P]orthophosphate from [^{32}P]phosphorylase *a*, according to Cohen *et al.* (35), in the presence of 2 nM okadaic acid and 0.2 mM inhibitor 2, respectively (35). These concentrations of okadaic acid and inhibitor 2 inhibited more than 95% of protein phosphatase activities when assayed on purified enzymes. PP2C activity was determined with ^{32}P-labeled casein as substrate (36) in the presence of 1 μM okadaic acid. Only 6% of initial PP2C activity was observed in Mg^{2+}-free buffer. PP2B (Promega, Madison, WI) activity was assayed spectrophotometrically at 410 nm with *p*-nitrophenyl phosphate as substrate (37). Protein phosphatase activity of alkaline phosphatase ALP (Sigma) was determined at pH 7.5 by measuring the release of [^{32}P]orthophosphate from phosphorylated casein in 50 mM Tris buffer containing 20 mM magnesium acetate. ALP activity was inhibited by 68% in the presence of 2 mM levamisole. All activities were expressed as pmol of phosphate release/min.

RESULTS

Discovery of Novel CFTR Activators—During the search for potential activators of the CFTR chloride channel (12, 13, 15, 18), we found a novel family of tricyclic compounds (Fig. 1*A*,

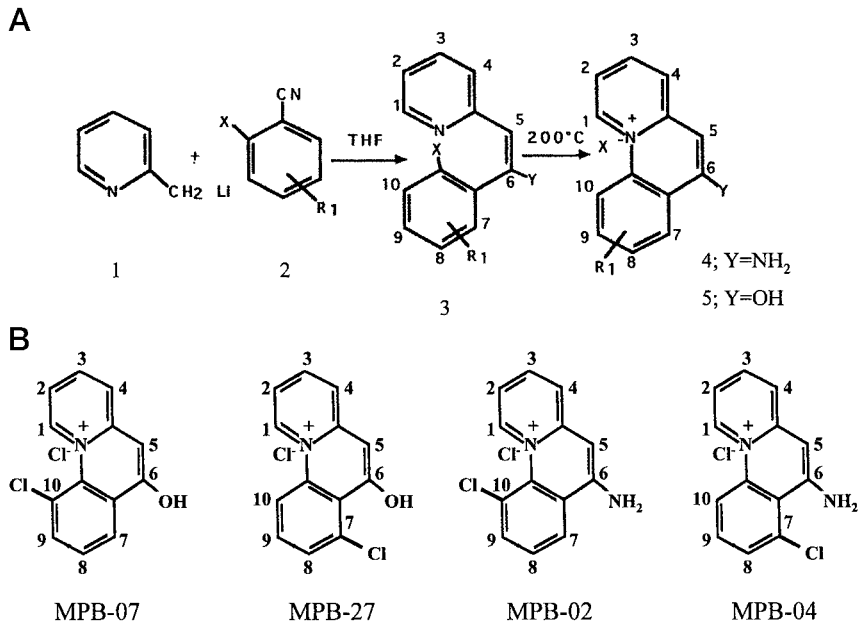

FIG. 1. **Benzo[c]quinolizinium compounds, synthesis, and structure.** *A*, scheme showing the experimental procedure for the synthesis of benzo[c]quinolizinium compounds. The condensation of 2-picolyllithium (*1*) and *ortho*-halogenobenzonitrile (*2*) gives the product *3*. Then thermocyclization at 200 °C generates two series of compounds depending on the presence of NH_2 (*4*) or OH (*5*). Other details are given under "Experimental Procedures." *B*, chemical structure for MPB-07 (6-hydroxy-10-chlorobenzo[c]quinolizinium chloride), MPB-27 (6-hydroxy-7-chlorobenzo[c]quinolizinium chloride), MPB-02 (6-amino-10-chlorobenzo[c]quinolizinium chloride), and MPB-04 (6-amino-7-chlorobenzo[c]quinolizinium chloride). Note the substitution at C-6 by OH (MPB-07 and MPB-27) or by NH_2 (MPB-02 and MPB-04).

compounds 4 and 5) (22). These compounds were synthesized, as described under "Experimental Procedures," by condensation of 2-picolyllithium (Fig. 1*A*, compound 1) and an *ortho*-halogenobenzonitrile (Fig. 1*A*, compound 2) to give compound 3 (Fig. 1*A*). Thermocyclization was then realized to obtain benzo[c]quinoliziniums (Fig. 1*A*, compounds 4 and 5). Among 15 different compounds, we selected four of them (Fig. 1*B*) for evaluation on CFTR chloride channel activity and epithelial secretory function: two compounds substituted at C-6 by OH and by a chlorine atom at C-10 (compound named MPB-07) or at C-7 (compound named MPB-27) and two MPB compounds substituted at C-6 by NH_2 and by a chlorine atom at C-10 (compound named MPB-02) or at C-7 (compound named MPB-04).

MPB Compounds Activate CFTR in Transfected CHO Cells—Fig. 2*A* shows typical whole cell currents and associated *I*/V plots (Fig. 2*B*) in the presence ($n = 5$) or absence ($n = 5$, control traces) of 10 μM forskolin in the bath indicating the presence of functional CFTR in this cell as previously demonstrated (38). As expected, the iodide efflux was significantly increased by forskolin (5 μM) or cpt-cAMP (500 μM) in CFTR(+) CHO but not in CFTR(−) CHO cells (Table I). Typical rates of ^{125}I efflux from forskolin-treated or control CHO(+) CFTR cells are shown in Fig. 2*C*.

We examined the effect of MPB compounds on the activation of CFTR chloride channels using CFTR(+) CHO cells. Fig. 3 shows whole cell currents (Fig. 3*A*) and associated *I*/V plots (Fig. 3*B*) in the presence ($n = 3$) or absence ($n = 5$, control traces) of 250 μM MPB-07 in the bath, demonstrating the presence of a linear chloride-selective current typical of CFTR in these cells. Similarly, the addition to the bath of MPB-07 (250 μM) caused a rapid increase in the rate of ^{125}I efflux to a peak rate 2 min after agonist addition (Fig. 3*C*). Fig. 4 shows the effect of MPB-27 that activated CFTR-dependent iodide efflux in a similar way. We also began a structure-function study and evaluated the effect on CFTR activity of chemical modifications within the MPB skeleton. To study the role of the OH group at C-6 in MPB-07 and MPB-27, we substituted it by NH_2 at the C-6 position, leaving unchanged the chlorine atoms at C-7 (compound named MPB-04, Fig. 4*A*) or at C-10 (compound named MPB-02, Fig. 4*A*). Surprisingly, both MPB-02 and MPB-04 failed to stimulate iodide efflux in CFTR(+) CHO cells (Fig. 4). A summary of iodide efflux data is given Table I. These results suggest that the nature of the group at the C-6 position of the MPB structure affects the potency of activation of these compounds.

The stimulation of the iodide efflux in CFTR(+) CHO cells by forskolin (5 μM), MPB-07 (250 μM), or MPB-27 (250 μM) was inhibited by ~90% using 100 μM glibenclamide (Table I) but not affected by 500 μM DIDS (Table I), indicating that CFTR was indeed the only chloride channel activated by these compounds. In CFTR(−) CHO cells, no stimulation of iodide efflux was observed in the presence of forskolin, cpt-cAMP, or MPB compounds (Table I).

To confirm whole cell and iodide efflux data, we also performed cell-attached patch clamp experiments. In control experiments using CFTR(+) CHO cells (*i.e.* in the absence of cAMP agonists), no spontaneous cell-attached CFTR channel activity was recorded ($n = 40$). As shown in Fig. 5, *A* and *B*, the addition to the bath of MPB-27 (250 μM) to a previously silent cell-attached patch caused progressive opening of multiple CFTR channels within 2 min. The analogue MPB-04 was found unable to activate CFTR (500 μM, $n = 4$, Fig. 5*C*). Fig. 6*A* shows the effect of MPB-07 (250 μM, $n = 6$) in the bath on cell-attached patches using CFTR(+) CHO cells. The activity of multiple CFTR chloride channel was again consistently observed in the presence of this derivative. MPB-02 again failed to open CFTR channels in cell-attached patch clamp experiments (Fig. 6*B*, 500 μM, $n = 4$). The linear current-voltage relationship and unitary conductance (6.9 ± 0.25 picosiemens, $n = 12$) were similar for both MPB-07 and MPB-27 compounds.

Effect of MPB on Murine Nasal Respiratory Cells—In contrast to CHO cells and human nasal cells (see below), prolonged exposure of murine respiratory cells to cAMP stimulants was necessary in order to observe CFTR currents. Fig. 7 shows examples of whole cell currents and associated *I*/V plots together with a summary of current densities for unstimulated wild type (WT) cells, WT and CF null cells pre-exposed for 24–30 h at 4 °C to the cAMP stimulants, and WT and CF null cells pre-exposed for the same time to 100 μM MPB-27. In the absence of agonists, only small currents were seen, which were not chloride-selective (Fig. 7*A1*). With the cAMP stimulants, we observed an essentially time-independent, nonrectifying conductance in 6 of 21 cells that had a reversal potential of −2.7 ± 1.9 mV, a value close to the equilibrium potential for Cl^- under these conditions (−5.7 mV). Note that only a subfraction of

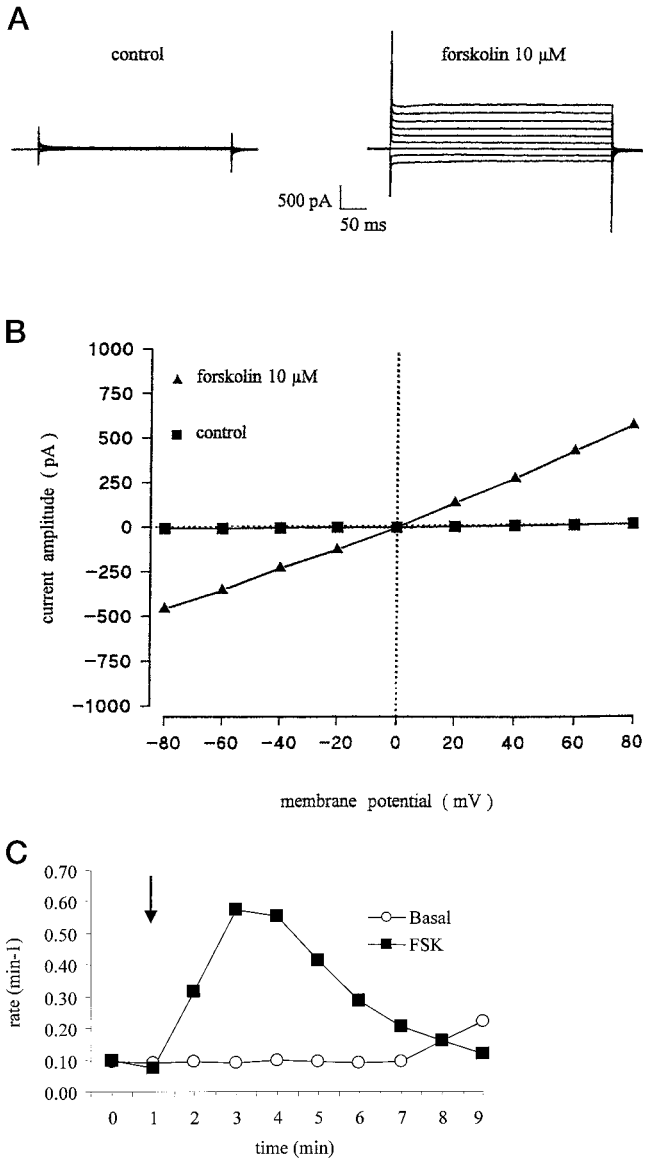

FIG. 2. **Characterization of forskolin-stimulated CFTR chloride channel activity by patch clamp and iodide efflux techniques in CFTR(+) CHO cells.** *A*, typical whole cell currents recorded for an unstimulated cell (noted control) and for a cell exposed to 10 μM forskolin in the bath. Holding potential was −40 mV. Voltages were pulsed to test potentials between −80 and +80 mV in 20-mV increments. *B*, corresponding *I*/*V* plots for data shown in *A*. *C*, activation of CFTR-mediated ^{125}I efflux by forskolin (5 μM, added at *arrow*). Rate of iodide efflux are plotted as a function of time.

murine respiratory cells responded to cAMP, suggesting that not all cells in the mouse nasal epithelium express CFTR. That these currents are Cl^--selective is further supported by the fact that Cl^- is the only permeant ion under the conditions used in these experiments. The Cl^- conductance had a current density of 10.2 ± 1.7 pA/pF and −10.2 ± 1.3 pA/pF when measured at the reversal potential ± 60 mV. Using 100 μM MPB-27, similar Cl^- currents were seen in 6 of 13 cells from two mice. These currents had a reversal potential of −4.5 ± 1.9 mV and a current density of 6.7 ± 2.6 pA/pF and −5.9 ± 1.4 pA/pF, data that are not significantly different to the cAMP-activated currents (either current density or frequency). Currents with similar properties were not present in nasal cells from transgenic CF null mice pre-exposed to either cAMP or MPB-27 (Fig. 7*D*), confirming that they are carried by CFTR channels. Overall, our experiments show that MPB-27 activates a chloride conductance with CFTR-like kinetics (time- and voltage-independent, linear *I*/V relationship) in nasal respiratory cells. Moreover, the respiratory cells remained viable after prolonged exposure to 100 μM MPB-27, and compared with stimulation with cAMP, a similar proportion of cells exhibited CFTR currents.

TABLE I
Effect of cAMP agonists and MPB compounds on the rate of ^{125}I efflux in CHO cells

Results are means ± S.E. The activity of CFTR channels was evaluated by use of the iodide efflux method. Experiments were performed with CFTR(−) CHO or CFTR(+) CHO cells. For each condition, the peak rate of iodide efflux (min^{-1}) is presented with the number of experiments in parentheses. *t* test: *, $p < 0.001$; NS, no significant differences. Statistical differences are given compared to the basal iodide efflux, except for experiments including inhibitors (inhibitor *versus* no inhibitor for a given agonist). Concentrations used are as follows: forskolin, 5 μM; cpt-cAMP, 500 μM; MPB-07, 250 μM; MPB-27, 250 μM, DIDS, 500 μM, glibenclamide (Glib.), 100 μM.

	Peak rate of iodide efflux	
	CFTR(−) CHO	CFTR(+) CHO
	min^{-1}	
Basal	0.080 ± 0.017 (8)	0.10 ± 0.026 (36)
Forskolin	(NS) 0.085 ± 0.015 (6)	*0.56 ± 0.28 (23)
cpt-cAMP	(NS) 0.091 ± 0.017 (6)	*0.54 ± 0.25 (15)
MPB-07	(NS) 0.082 ± 0.027 (6)	*0.52 ± 0.18 (13)
MPB-27	(NS) 0.086 ± 0.020 (6)	*0.45 ± 0.17 (10)
MPB-02	(NS) 0.079 ± 0.035 (4)	(NS) 0.12 ± 0.020 (4)
MPB-04	(NS) 0.085 ± 0.040 (4)	(NS) 0.11 ± 0.017 (4)
Forskolin + DIDS		(NS) 0.51 ± 0.15 (4)
MPB-07 + DIDS		(NS) 0.53 ± 0.16 (4)
MPB-27 + DIDS		(NS) 0.48 ± 0.09 (4)
Forskolin + Glib.		*0.11 ± 0.05 (4)
MPB-07 + Glib.		*0.14 ± 0.04 (4)
MPB-27 + Glib.		*0.13 ± 0.03 (4)

Effect of MPB on Short Circuit Current in Human Nasal Cells—The MPB-07 compound was tested on polarized preparations of human nasal epithelial cells after blocking the epithelial Na^+ channel with amiloride (10 μM). These cells express CFTR as indicated by the presence of a glibenclamide-sensitive cAMP-dependent current. Indeed, the stimulation with cpt-cAMP (100 μM) increased the short circuit current by 22.4 ± 3.4 $\mu A/cm^2$, $n = 3$ (Fig. 8*A*). This current was completely blocked by 500 μM glibenclamide (Fig. 8*A*). MPB-07 was applied in the apical solution at increasing concentrations (from 1 to 200 μM). This compound elicited stable increases of the short circuit current in a dose-dependent fashion (Fig. 8*B*). At 200 109 μM, the current induced by MPB-07 was 12.9 ± 0.9 109 $\mu A/cm^2$ ($n = 4$). Glibenclamide completely blocked this current (Fig. 8*B*).

Effect of MPB on Mucin Secretion, cAMP, and ATP Levels in Rat Submandibular Acinar Cells—MPB-07 was tested on secretion of mucins in a polarized preparation of rat submandibular acini, which express CFTR (6, 31). The actions of MPB-07 have been compared with that of physiological stimulation evoked by the β-adrenergic agonist, isoproterenol. Table II shows that the compound MPB-07 significantly stimulated mucin secretion from rat submandibular acini, although to a much lesser extent than a maximally effective concentration (10 μM) of the β-adrenergic agonist, isoproterenol. In the presence of isoproterenol, MPB-07 did not further increase mucin secretion (MPB-07 (100 μM) plus isoproterenol (10 μM): 107.4 ± 9.3%, $n = 4$ of isoproterenol alone; MPB-07 (500 μM) plus isoproterenol (10 μM): 104.8 and 96.9%, $n = 2$ of isoproterenol alone), indicating that MPB-07 was increasing mucin secretion by the same final common mediator as isoproterenol, which we have shown to be CFTR (6, 31). MPB-07 did not increase intracellular cAMP, suggesting a direct action on CFTR. MPB-07 did not change cellular ATP levels over a 60-min incubation period, nor did it increase lactate dehydrogenase release (data not shown),

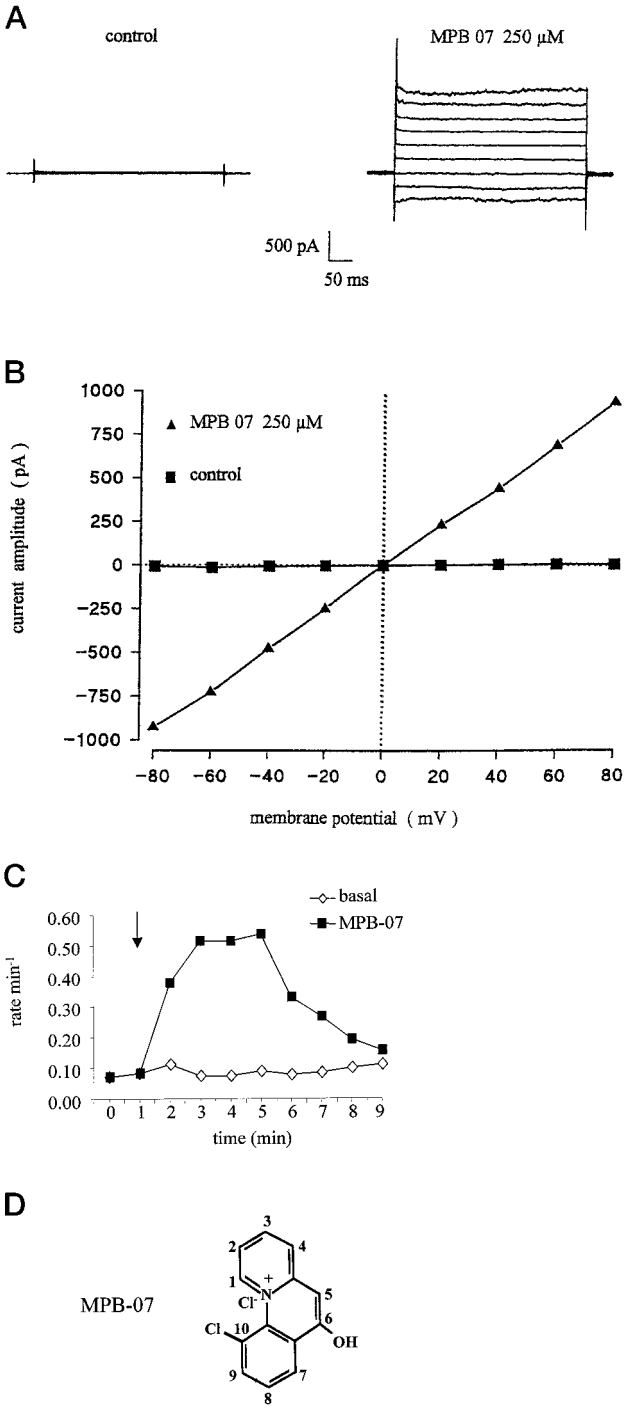

FIG. 3. **Whole cell patch clamp and iodide efflux analyses examining the effect of MPB-07 in CFTR(+) CHO cells.** *A*, typical whole cell currents recorded for an unstimulated cell (labeled *control*) and for a cell exposed to 250 μM MPB-07 in the bath. Holding potential was −40 mV. Voltages were pulsed to test potentials between −80 and +80 mV in 20-mV increments. *B*, corresponding *I*/V plots for data shown in *A*. *C*, activation of CFTR-mediated ^{125}I efflux by 250 μM MPB-07. Rate of iodide efflux are plotted as a function of time. MPB-07 was added as indicated by the *arrow*. *D*, chemical structure for MPB-07.

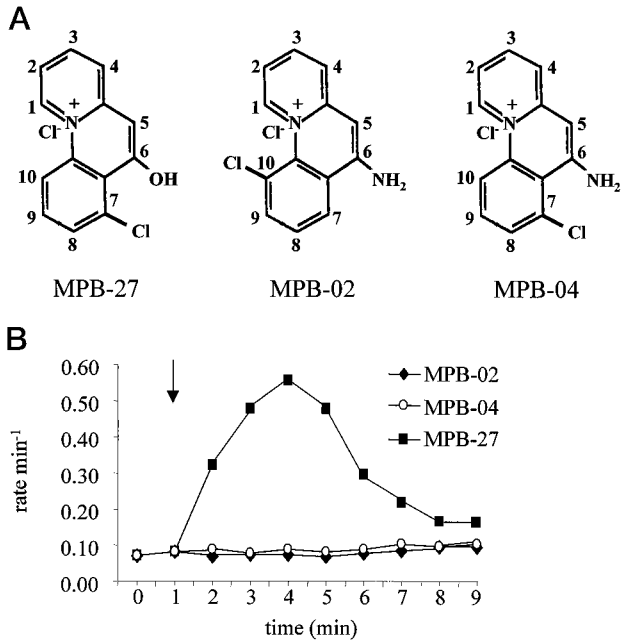

FIG. 4. **MPB-dependent iodide efflux in CFTR(+) CHO cells.** *A*, chemical structure for MPB-27, MPB-02, and MPB-04. *B*, experiments showing the rate of iodide efflux plotted as a function of time when MPB-02, MPB-04, or MPB-27 (500 μM) were added (*arrow*). Note that only MPB-27 stimulates the rate of iodide efflux.

indicating that it had no effect on cell viability or leakage of cytoplasmic contents.

Effect of MPB on CFTR Chloride Channel Activity of, and on the Secretion of Protein by, the Human Tracheal Gland Cell Line MM39—We characterized the activation of CFTR chloride channels by cAMP agonists in the human tracheal gland cell line MM39 (33). Fig. 9 shows that MPB-07 (250 μM, $n = 3$) in cell-attached patch-clamp experiments, caused the activation of multiple CFTR chloride channels (Fig. 9*A*) with an average unitary conductance of 9 ± 2.1 picosiemens, $n = 3$ (Fig. 9*B*), consistent with previous data obtained using cAMP agonists (33).

It is known that the secretagogue agent ATP, proposed for CF therapy, acts on the human tracheal gland cell line MM39 by increasing protein secretion (32). This effect is mediated by cAMP generation and through calcium mobilization (32). We examined the effect of MPB-07 on the secretion of the SLPI by MM39. The results are expressed as the percentage of the SLPI secreted by the assay to the SLPI secreted in control experiments. Fig. 9*C* shows that ATP (100 μM, $n = 8$) or MPB-07 (100 μM, $n = 8$) has a similar stimulatory effect on secretion of SLPI. The combination of MPB-07 and ATP (both at 100 μM, $n = 8$) showed additive effects. The responses were similar to that predicted by summation of the effects of each agent added independently. The response to MPB-07, ATP, or ATP plus MPB-07 was 59 ± 11 ($p < 0.01$), 52 ± 15 ($p < 0.01$) and 93 ± 15% ($p < 0.01$) above control, respectively. Thus, in human tracheal gland cells, MPB compounds are able not only to activate CFTR but are also able to stimulate the secretion of a protein involved in the antiproteolytic (39) and antibacterial (40) defense of the airway.

Effect of MPB on cAMP and ATP Levels in CHO Cells—We tested the possibility that activation by MPB might be due to elevation of cAMP. In resting CFTR(+) CHO cells, the cellular cAMP content was 18.3 ± 2.08 pmol of cAMP/mg of protein, $n = 9$ (Table III). As expected, forskolin (5 μM, $n = 9$) increased the cAMP level measured after 5 min (Table III). In contrast, the corresponding cAMP level determined in the presence of MPB-07, MPB-27, MPB-02, and MPB-04 ($n = 9$ for all compounds at 500 μM) was not increased compared with the basal level (Table III). These results argue against a role of cAMP in mediating the effect of MPB compounds on CFTR. In addition, these results are comparable with that observed in submandibular acinar cells. We also measured the effect of MPB compounds on the ATP content of CFTR(+) CHO cells. In resting cells, the

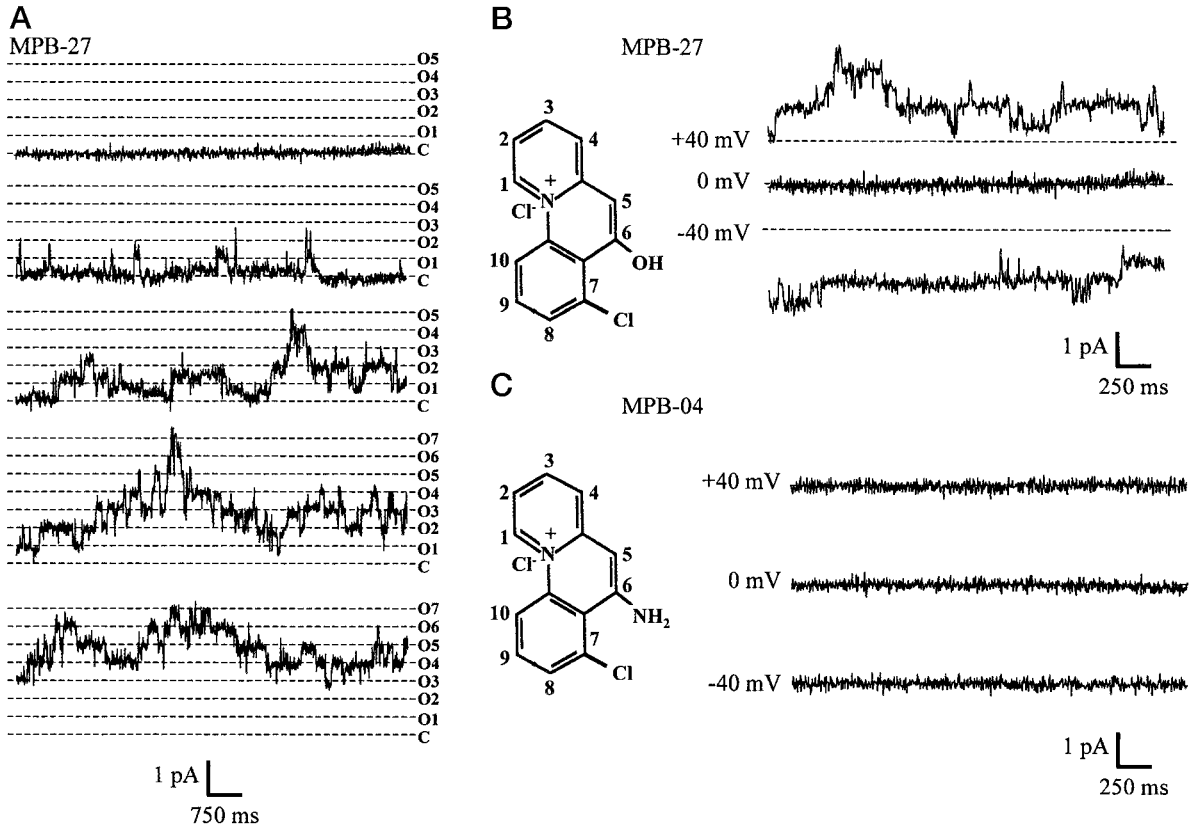

FIG. 5. **Single CFTR chloride channel activation by MPB-27 but not by MPB-04 in CFTR(+) CHO cells.** *A*, continuous cell-attached recording obtained on a CHO cell stably expressing CFTR showing the activation of CFTR chloride channels by 250 μM MPB-27 in the bath. The compound was added at the beginning of the recording (*top trace*). Note the progressive opening of up to seven channels. The levels of channel currents are noted to the *right* of each *trace* (*C*, closed state; *O*, open state). *B* and *C*, representative recordings at various patch potentials as indicated, in the presence of MPB-27 (*B*) or MPB-04 (*C*), both at 250 μM in the bath. For clarity, the chemical structure of the respective compound used is shown. Note that with MPB-04 no channel activity was observed.

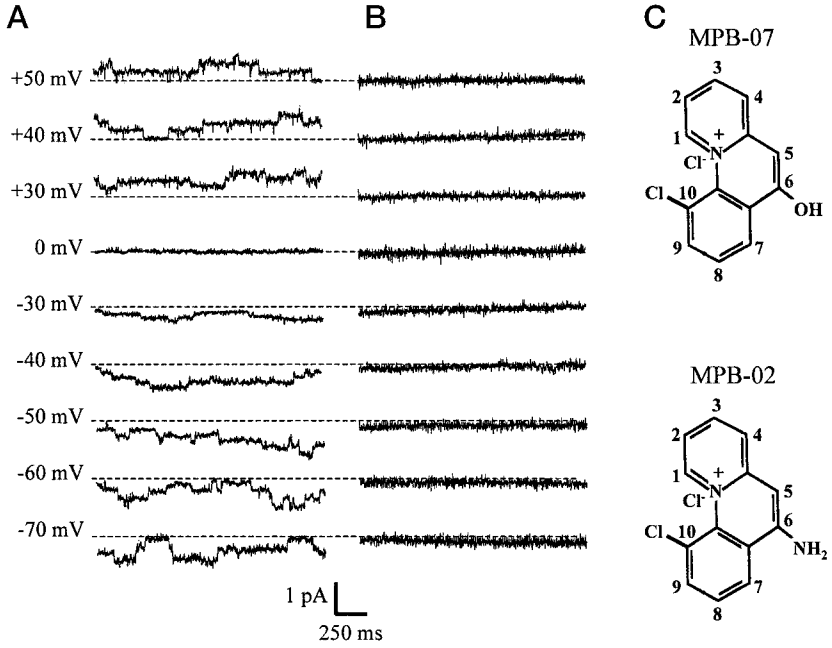

FIG. 6. **Single CFTR chloride channel activation by MPB-07 but not by MPB-02 in CFTR(+) CHO cells.** *A* and *B*, representative recordings in cell-attached configuration at various patch potential of CFTR chloride channels in the presence of MPB-07 (*A*) or MPB-02 (*B*), both at 250 μM in the bath. Note that with MPB-02 no channel activity was observed. *C*, the chemical structure of the respective compound used is shown.

level of ATP was 51 ± 5 nmol/mg of protein (n = 6). At a concentration of 500 μM, MPB-07 and MPB-27 have no effect on the ATP content of CHO cells. These data also suggest that MPB drugs did not stimulate CFTR channels through modulation of cellular ATP.

Effect of MPB on Protein Phosphatase Activities—Protein phosphatase inhibition has been shown to activate CFTR channels in a variety of cells including CHO cells (12, 15, 41–43). To test whether our compounds might activate CFTR through the inhibition of endogenous phosphatases, we measured in CFTR(+) CHO cells the activity of the principal protein phosphatases (Table IV) previously described to regulate CFTR (12,

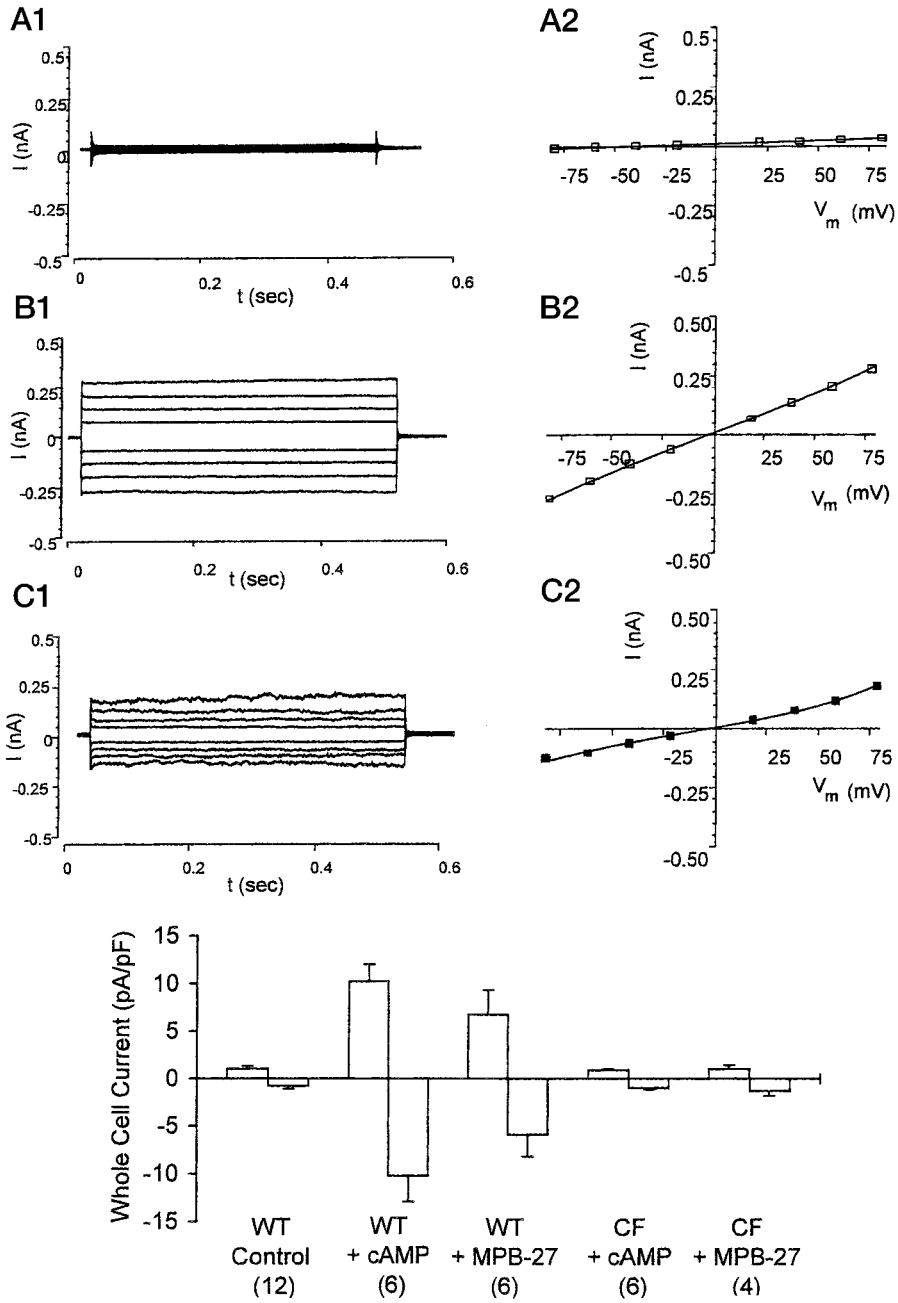

FIG. 7. **Activation of CFTR currents by cAMP and MPB-27 in ciliated respiratory cells from the nasal epithelium of wild type and CF null mice.** *A1*, *B1*, and *C1*, typical whole cell currents recorded by holding the membrane potential at 0 mV and pulsing to voltages in a range of ±80 mV in 20-mV steps for an unstimulated cell (*A1*), a WT cell pre-exposed to the cAMP stimulants (*B1*), and a WT cell pre-exposed to 100 μM MPB-27 (*C1*). *A2*, *B2*, and *C2*, corresponding *I*/V plots for data in *A1*, *B1*, and *C1*. *D*, summary of current densities (pA/pF) in unstimulated wild type cells (*WT Control*), WT cells pre-exposed to the cAMP stimulants (*WT + cAMP*), WT cells pre-exposed to 100 μM MPB-27 (*WT + MPB-27*), CF null cells pre-exposed to the cAMP stimulants (*CF + cAMP*), CF null cells pre-exposed to 100 μM MPB-27 (*CF + MPB-27*). Cells were pre-exposed to either cAMP stimulants or MPB-27 for 24–30 h at 4 °C (see "Experimental Procedures").

41, 42). Table IV shows that MPB-07 had no effect on the endogenous PP1, PP2A, PP2C, and alkaline phosphatase activities. Similarly, the PP2B phosphatase was not affected by the compound (Table IV).

DISCUSSION

Pharmacology of CFTR is still poor, and only a few compounds with low specificity have been shown to modulate its activity. Therefore, to test new products, we have developed a collaboration with several European laboratories. Selected compounds that arose from our screening strategy were evaluated independently in these laboratories, and results are presented in this report.

Novel Activators of CFTR Chloride Channels—We have generated by chemical methods a series of substituted MPB compounds, among them MPB-27 and MPB-07, which we show to be potent and selective activators of the CFTR chloride channel in all of the cell models tested in this study (*i.e.* in CHO cells stably expressing wild type CFTR, in human tracheal gland MM39 cell lines, in native respiratory cells isolated from wild type mice, in rat submandibular acinar cells, and in human nasal epithelial cells). Activation of CFTR by MPB compounds is shown to be cAMP- and ATP-independent, glibenclamide-sensitive and DIDS-insensitive, two well established properties of CFTR (4, 44), indicating the specificity of these drugs for CFTR. The successful activation of CFTR chloride current in murine and human respiratory cells is of particular interest, since it proves that MPB compounds are good candidates for the pharmacological activation of CFTR in airways.

MPB Drugs Stimulate the Antibacterial Function of Human Tracheobronchial Gland Cells—Interestingly, we have demonstrated in this study that beside their effect on CFTR chloride channels, MPB drugs may stimulate the defense protein secretion of human tracheobronchial gland cells. Human tracheal glands are considered as the main secretory structure in the bronchotracheal tree and are among the human airway cells that highly express CFTR (45). We studied the human tracheal

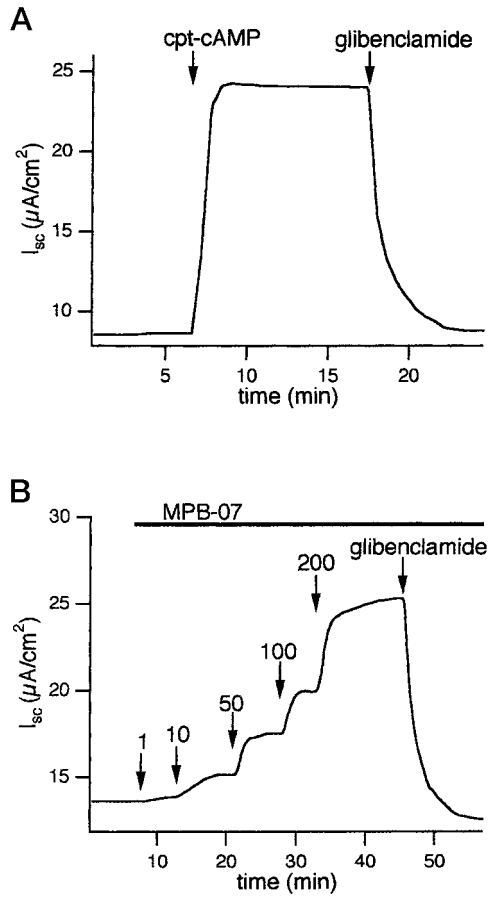

FIG. 8. **Short circuit current (I_{sc}) measurements on human nasal epithelial cells.** The *figure* depicts short circuit recordings in two representative experiments. In both cases, the epithelial Na^+ channel was previously blocked with 10 μM amiloride (not shown). *Trace A* represents the response to the apical and basolateral application of cpt-cAMP (100 μM) and the inhibition caused by glibenclamide (500 μM). *Trace B* shows the effect of increasing concentrations (in μM) of MPB-07 in the apical solution. The effect of 500 μM glibenclamide is also shown.

TABLE II

Effect of MPB-07 on mucin secretion, cyclic AMP, and ATP levels in rat submandibular acinar cells

Results are means ± S.E.; $n = 6$. Mucin secretion and ATP content were measured after 60 min, and cAMP was measured after a 5-min incubation. **, $p < 0.002$; *, $p < 0.05$ for difference from no addition as assessed by Student's t test.

Addition	Mucin secretion	Cyclic AMP	ATP content
	% basal	*pmol/mg protein*	*nmol/mg protein*
None	100 ± 3.4	8.6 ± 0.7	3.9 ± 0.2
Isoproterenol (10 μM)	**1025.6 ± 110.6	**447.6 ± 71.1	3.2 ± 0.3
MPB-07 (100 μM)	*122.6 ± 8.2	9.4 ± 0.9	3.6 ± 0.2
MPB-07 (500 μM)	*146.4 ± 15.5	9.5 ± 1.1	4.3 ± 0.3

gland cell line MM39 because it has retained the physiological characteristics of the genuine cells, namely CFTR expression, high capacity of ionic transport (33), and constitutive and stimulated secretion of proteins highly involved in the defense mechanism of the bronchotracheal tree (32). These are SLPI, lactoferrin, and lysozyme participating in the antibacterial activity of the lung. SLPI is the major antiprotease of the epithelium of the upper respiratory tract providing protection against neutrophil elastase (39). The effects of drugs, active on CFTR, on the secretion of proteins involved in lung defense is therefore of primary importance. We have shown here that MPB compounds are able to activate CFTR and to stimulate SLPI secretion, suggesting that CFTR is involved in the secretory process. Indeed, a defect in protein secretory mechanisms is a hallmark of CF gland cells (5, 46). We may speculate that MPB stimulates the secretion of SLPI by a mechanism different from that of ATP and possibly through the direct activation of CFTR, which in turn promotes this secretory pathway.

MPB Compounds and Mucin Secretion—In keeping with these data, we have shown that MPB-07 increased CFTR-mediated mucin secretion in rat submandibular acinar cells and that this effect does not involve cAMP. These results are in line with the preceding data of Lloyd Mills *et al.* (6) on these same cells, showing that the incorporation of anti-CFTR antibodies into the cells inhibited β-adrenergic-stimulated mucin secretion (6). Moreover, it has been shown that the transfection of cDNA for wild type CFTR into CFPAC-1 cells, which conferred cAMP-dependent regulation of a Cl^- conductance (47), restored the defective ATP-induced mucin secretion observed in CF cells (48). Similarly, adenovirus-mediated gene transfer of CFTR to immortalized CF human tracheal epithelial cells restored defective cAMP-dependent secretion not only of chloride but also of glycoconjugates (49). Taken together, these observations suggest that the presence of a functional CFTR protein is necessary for the regulation of macromolecule secretion. This also suggests that the MPB compounds are useful not only as CFTR Cl^- channel activators but also as stimulators of CFTR-mediated protein secretion. It also strengthens the hypothesis that CFTR controls the secretion of proteins and/or mucins in epithelial cells.

Structure-Activity of MPB Compounds—To complement these studies, we also began a structure-activity analysis of the MPB family to gain information on the structural components important for CFTR opening. In a first approach, we have studied the effect of chemical modification of the OH group at the C-6 position and generated two different series of compounds with OH or NH_2 at C-6. Replacement of OH by NH_2 abolishes the ability of MPB to activate CFTR, since compounds substituted at C-6 by OH (MPB-07 and MPB-27) but not by NH_2 (MPB-02 and MPB-04) open CFTR. Within the OH-substituted series, the position of the chlorine atom at C-7 (MPB-27) or C-10 (MPB-07) generated two apparent equivalent activators of CFTR. These data strongly indicate that MPB activation of CFTR depends not only on the position, but also on the nature of the substituent group. We are now further investigating the structure-activity relationship of these chemicals to determine the structural basis for specificity and potency of MPB derivatives as activators of CFTR.

In conclusion, we report the discovery of a family of substituted benzo[c]quinolizinium compounds as novel activators of the CFTR chloride channel and of CFTR-mediated protein secretion. These compounds activate CFTR in a variety of cell models, including recombinant and epithelial cells from humans, rats, and mice, without affecting the intracellular levels of cAMP and ATP or the activity of various phosphatases. These drugs are easy tools to use in laboratories, since we show that all of the classical techniques commonly used to study CFTR channel function (whole cell and single patch clamp recordings, iodide efflux, short circuit measurement) are suitable. Moreover, several lines of evidence suggest that these drugs specifically activate CFTR without an effect on other chloride channels. For example, in a comparison of CF null and wild type mice, CFTR appears to be the only chloride conductance activated by MPB compounds. Similarly, in MM39 and human nasal cells, no other chloride conductance appeared to be activated by MPB. The specificity of MPB compounds as CFTR activators is also strengthened because they have no apparent effect on intracellular cAMP and ATP. Finally, MPB

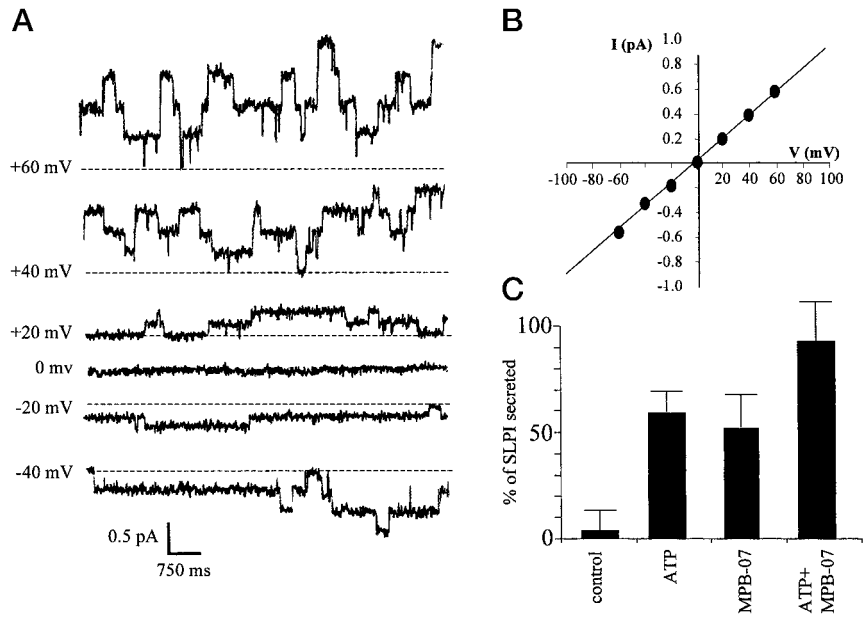

FIG. 9. **Stimulation of single CFTR chloride channel activity and SLPI secretion by MPB-07 on human tracheal gland cells.** *A*, typical current traces at the indicated patch potentials from a cell-attached recording from MM39 cells activated by 250 μM MPB-07 in the bath solution. *Dashed lines* indicate the closed state of the channels. *B*, plots of current-voltage data displayed in *A*. *C*, secretion of the secretory leukoproteinase inhibitor SLPI by MM39 cells in the presence of ATP (100 μM), MPB-07 (100 μM), or ATP + MPB-07 (both at 100 μM) *versus* control (no drugs added). The results are expressed as the percentage of the SLPI secreted in the assays above that secreted in control experiments, $n = 8$ for each condition.

TABLE III
Effect of MPB-07 on cAMP level in CHO cells

Results are means ± S.D. for $n = 9$. Intracellular cAMP content in resting CFTR(+) CHO cells and during stimulation with forskolin (5 μM) or MPB (500 M).

Addition	Cyclic AMP
	pmol/mg protein
None	18.3 ± 2.1
Forskolin	762 ± 198
MPB-07	17.6 ± 6.3
MPB-27	27.6 ± 4.1
MPB-02	27.4 ± 5.1
MPB-04	59.7 ± 16

TABLE IV
Effect of MPB-07 on protein phosphatase activities in CHO cells

Results are means ± S.D. Protein phosphatase activities were assayed in triplicate in two different experiments that gave similar results. One of these experiments is presented. All values are activities in incubation medium except for PP2B, where activity is multiplied by 10^{-3}.

Phosphatase	Activity	
	Control	MPB-07 (500 μM)
	pmol phosphate/min	
PP1	2.39 ± 0.08	2.64 ± 0.04
PP2A	1.39 ± 0.35	1.67 ± 0.35
PP2B	1.78 ± 0.15	1.76 ± 0.21
PP2C	0.42 ± 0.02	0.44 ± 0.05
ALP[a]	0.16 ± 0.01	0.16 ± 0.03

[a] Alkaline phosphatase.

compounds are potentially very useful drugs because they show an apparent low cellular toxicity. They could be used to clarify the role of CFTR in protein and mucin secretion as well as in the overall chloride conductances of several tissues including heart, nephron, and airway. They also may help to investigate the role of CFTR as regulator of other chloride channels in epithelia.

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Development of Substituted Benzo[c]quinolizinium Compounds as Novel Activators of the Cystic Fibrosis Chloride Channel

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